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RESEARCH ARTICLE

HIRSCHSPRUNG'S DISEASE – OUR EXPERIENCE TO DETECT IT ON TABLE USING RAPID ACETYLCHOLINE ESTERASE STAINING METHOD IN A RESOURCE RESTRICTED TERTIARY CARE HOSPITAL SETUP

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ABSTRACT

Background and objective: Hirschsprung's disease is a disease that occurs in a newborn child with an incidence of one in 5000 live births with a male predominance. In our setup for the diagnosis of Hirshcsprung's disease, haematoxylin and eosin staining in a formalin fixed tissue was routinely practiced. As a novel diagnostic procedure, rapid on table acetylcholine esterase stain was tried. It is a diagnostic accuracy test done with the following objectives: 1.To perform acetylcholine esterase and rapid hematoxylin and eosin staining in the frozen section of rectal biopsy as a part of intra operative consultation. 2 To correlate routine formalin fixed, hematoxylin and eosin staining results with rapid methods. 3. Feasibility of these procedures in our setup.

Method: All infants who had not passed meconium since birth and who had symptoms of intestinal obstruction were screened by pediatric surgeons. As a part of intra-operative consultation, both frozen section and formalin fixation were done in the received specimen. In the frozen section, acetylcholine esterase staining and rapid haematoxylin and eosin staining was done. In the formalin fixed specimen routine haematoxylin and eosin was done.

Results: All the three stains- acetylcholine esterase, rapid hematoxylin and eosin and hematoxylin and eosin in a formalin fixed specimens gave equal results. When rapid methods were compared with the routine formalin fixed specimen haematoxylin and eosin staining method the turnaround time was very much reduced. By rapid staining, intra-operative consultation facilitated single stage Duhamel pull- through. Repeat surgery was avoided as both the diagnosis and the level of colon having ganglion cells were assessed simultaneously. As a result, hospital stay was reduced.

Conclusion: In this diagnostic study done on twenty cases of suspected Hirschsprung's disease, the diagnostic efficacy of rapid methods correlated with that of the routinely processed tissue sections stained with haematoxylin and eosin. The turnsaround time for the rapid tests were less. This facilitated on table diagnosis, reduced the need for repeat surgery, improved the outcome of surgery and, there by reduced the duration of hospital stay.

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INTRODUCTION

Hirschsprung's disease (HSD) is a multi-genetic neurocrisopathic disorder of newborns and children. The incidence is estimated to be one in 5000 newborns, according to Badner *et al* and Parisi & Kapur (Badner *et al*., 1990; Kapur, 2001). But it varies between the racial groups. In Asians the incidence ratio is 2.8 per 10,000 live births (Torfs, 1998). There is a male predominance, male to female ratio being 4:1. This is less evident in patients with long segment aganglionosis and Down syndrome. Hirschsprung's disease has a sporadic occurrence. In about 20% of the sporadic cases, RET genetic mutation is present (Edery *et al*., 1994). The gold standard in the diagnosis of HSD is the demonstration of absent ganglion cells in the histopathological sections of rectal biopsy (Natarajan *et al*., 2002).

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In 80% of the cases the aganglionosis involves the recto-sigmoid colon, in 11 to 26% the left colon, and in 5 to 15% the entire colon (Bax K. Duhamel, 2006). Usually, full thickness rectal biopsy specimen is examined for the evaluation of ganglion cells. The alternative is suction biopsy of the rectal mucosa, sectioning with cryostat and rapid acetylcholine esterase [ACHE] staining. ACHE stains on frozen sections had given best results in diagnosing HSD according to Coffin CM, *et al*. Meier-Ruge W *et al* and Qualman *et al*. (Coffin CM, *et al*., 2005 Meier-Ruge *et al.,*1972; Qualman *et al.,* 1999). Increase in acetylcholine esterase in the affected tissue is the pathophysiology of this disease (Schofield *et al*., 1990; Bagdzevicius *et al*., 2007; Ghosh and Griffiths, 1998). And acetylcholine esterases do not stain the normal nerve fiber. This concept is used in identifying this disease (Kleinhaus *et al*., 1979).

Before this study was attempted, the procedure followed in our hospital for patients suspected of Hirschsprung's disease were colostomy and formalin fixed full thickness rectal biopsy for ascertaining the diagnosis. Which of course takes time for the processing and later, after confirmation, Duhamel pull through was done. Frozen section and acetylcholine esterase staining was introduced in our histopathological lab in order to know the feasibility of this technique to diagnose accurately and to reduce the turnaround time of the rectal biopsy specimens received. Both confirmations of the diagnosis and the presence of ganglion cells from the cut ends were to be made at the same time. This study was done to evaluate the diagnostic accuracy of rapid on table staining method.

MATERIALS AND METHODS

During our study period (November2011-2013), twenty rectal mucosal biopsies were received from the Department of Paediatric surgery. Rectal biopsy from the children aged 4 days to 12 years suspected to have Hirschsprung's disease were received (Table 1).

Inclusion criteria

- All infants with symptoms suggestive of Hirschsprung's disease – Late passage of meconium, abdominal distension, bilious vomiting, and Hirschsprung's-associated entero-colitis were included.
- When investigated with contrast enema, they should reveal features of the narrow rectal segment, transition zone and proximal bowel dilation.
- Patients should be screened and selected by the Department of Pediatric surgery and their rectal biopsies sent to the Department of Pathology for evaluation
- Biopsy specimens should be taken 2-3cm proximal to dentate line.

Exclusion criteria

- If biopsy specimens contain epithelium of the anal canal.
- Inadequate specimen.
- Specimens that were not received in saline.

We were pre- informed and the biopsy specimens were transported to the laboratory in a saline soaked gauze or in saline. Specimens from the rectal mucosa 2-3 cm above the dentate line and specimens from various levels of the intestinal segment for assessment of ganglion cells were sent. They were labeled accordingly. Specimens were accompanied by a requisition form with detailed history, investigation and levels from where the biopsies were taken. The specimen was then washed to remove the blood, as red cells would take up the ACHE stain and cause background staining. Frozen sections of 10 µm thickness were taken after labeling the slides. Slides were stained with acetylcholine esterase and with rapid haematoxylin and eosin (H&E) method. Only part of the tissues were used for frozen section. The rest of the tissues were fixed in formalin and it was processed routinely and stained with haematoxylin and eosin. Acetylcholine esterase gives dark green color to the nerves when seen under light microscopy.

The principle behind the ACH stain

The thiocholine product produced react with ferricyanide and reduces it to ferrocyanide.It precipitates with copper to form copper ferrocyanide. This produces the dark green color according to Karnowsky and Roots (Karnowsky and Roots, 1964).

Interpretation of the ACHE stains

Presence of hypertrophic nerve bundles and absent ganglion cells are the criteria used for the diagnosis.

- Nerve fibers are stained greenish black
- Ganglion cells are negatively stained, (Fig 1)

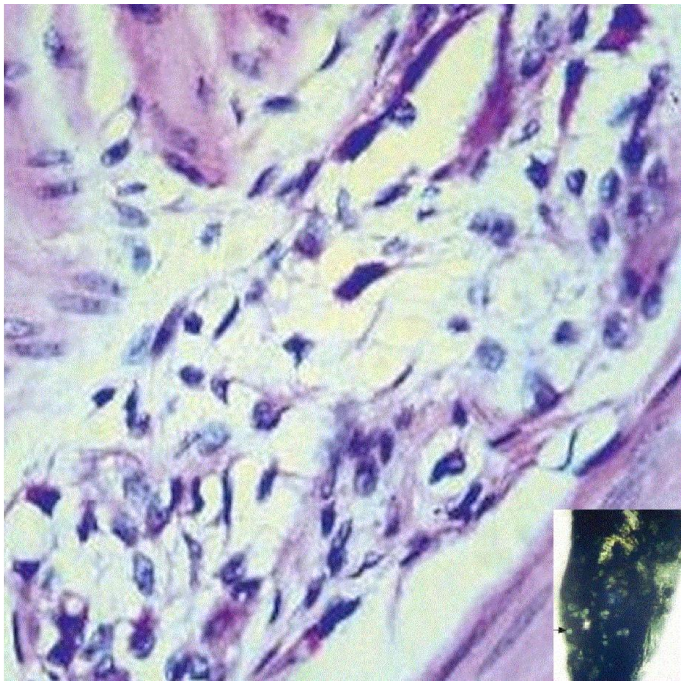

Fig. 1. Shows ganglion cells [H&E,40x].Inset shows ganglion cells are negatively stained with acetylcholine esterase[40x]

Four patterns are observed with ACHE staining based on level of stained nerve fibers. Nerve fibers and red blood cells provide the positive internal control.

Pattern A: Nerve plexus between the crypts in lamina propria, submucosa and muscularis mucosa stain positive with acetylcholine esterase, (Fig 2 & 3).

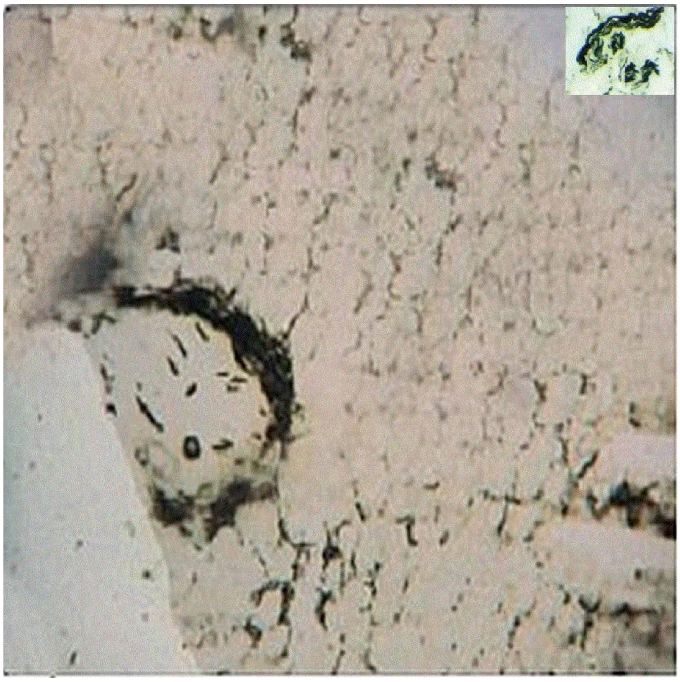

Fig. 2. Shows nerve plexus between the crypts in lamina propria, submucosa and muscularis mucosa stained positive with acetylcholine esterase- Pattern A.[4x].Inset shows hypertrophic nerve bundle [ACH,40x]

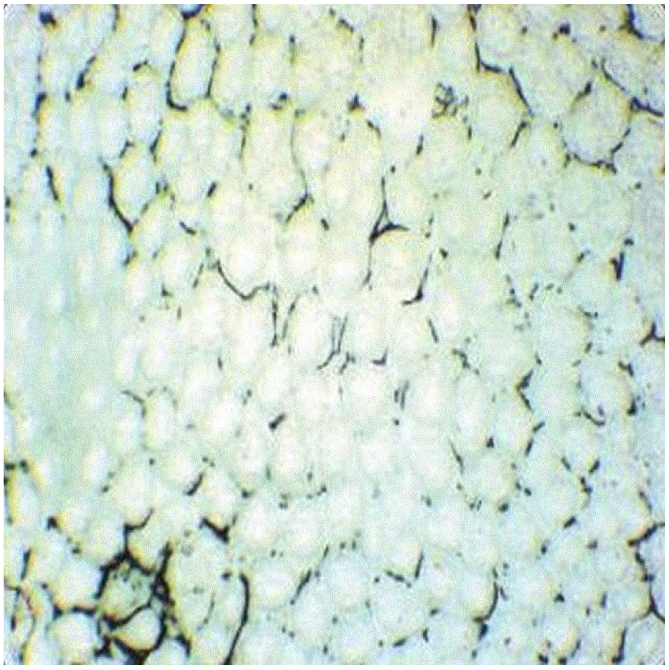

Fig. 3. Shows nerve plexus between the crypts in lamina propria, submucosa and muscularis mucosa stained positive with acetylcholine esterase- Pattern A[40x]

Pattern B: Nerve plexus at the base of the crypts, submucosa and muscularis mucosa stain positive with acetylcholine esterase, (Fig 4).

Equivocal pattern: Nerve fibers in the submucosa alone stained by acetylcholine esterase and absent ganglion cells.

Negative pattern: No staining identified

Interpretation of results of H & E stained sections

Positive for Hirschsprung's disease: Presence of hypertrophic nerve bundles in the submucosa and muscularis propria and absent ganglion cells, (Fig 5 & 6- Rapid H & E and Routine H&E respectively).

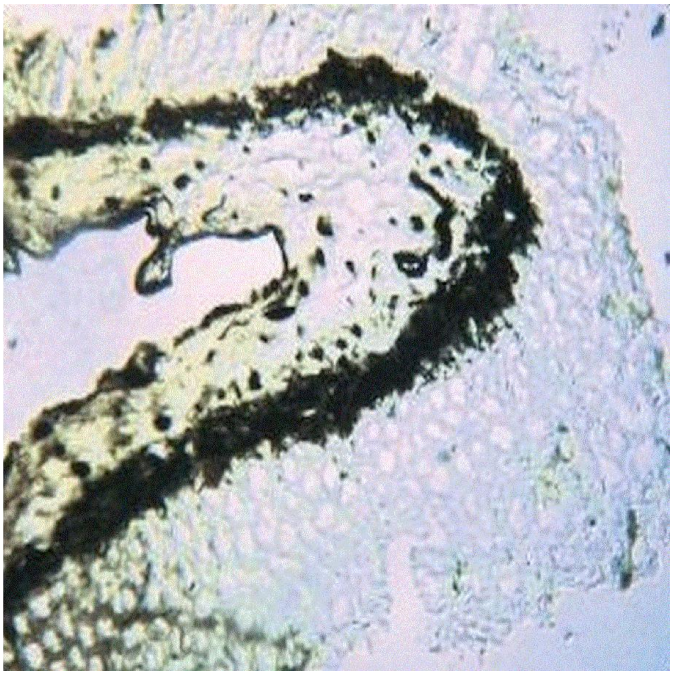

Fig. 4. Shows nerve plexus at the base of the crypts, submucosa and muscularis mucosa stained Positive with acetylcholine esterase- Pattern B[4x]

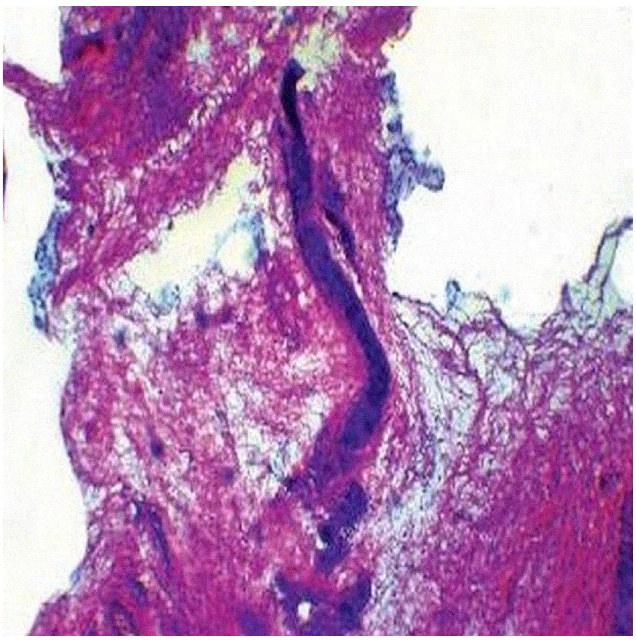

Fig. 5. Shows presence of hypertrophic nerve bundles in the muscularis propria and absent ganglion cells,[Rapid H&E, 40x]

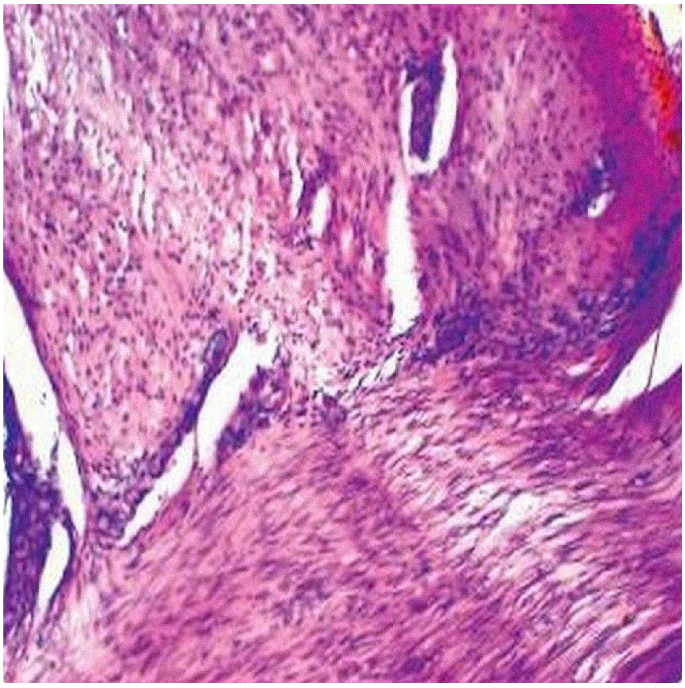

Fig. 6. Shows Presence of hypertrophic nerve bundles in the muscularis propria and absent ganglion cells,[Routine H&E,40x]

Transition zone: Ganglion cells are distributed unevenly along the circumference with hypertrophic nerve bundles. Negative for Hirschsprung's disease: Ganglion cells are present.

RESULTS

Over a two-year study period(November 2011-2013), twenty rectal mucosal biopsies from the children aged 4 days to 12 years suspected of Hirschsprung's disease were received from the Department of Pediatric surgery (Table 1).

Table 1. Age and sex distribution of patients suspected with Hirschsprung's disease

Age group(Years)	Male	Female
0-1	10(50%)	2(10%)
1-5	6(30%)	1(5%)
6-12	1(5%)	Nil

Positive results were obtained in all the three methods, namely, acetylcholine esterase stained sections, rapid haematoxylin and eosin stained sections and in those sections that were formalin fixed, routinely processed and stained with haematoxylin and eosin stain (Table2).

Table 2. Cases positive with ACH, rapid H&E and routine H&E stains

Positive with ACH stain	Positive with rapid H&E	Positive with routine H&E
16	16	16

There was no difference in the diagnostic sensitivity within these three methods used. Ghosh and Griffithshad used both haematoxylin and eosin and acetylcholine esterase to diagnose Hirschsprung's disease (Ghosh and Griffiths, 1998). When analyzing the results of their sensitivity, all the stains were 100% sensitive with 95% confidence interval being 79.41% to 100.00%. Specificity was 100%, 95% confidence interval being 39.76% to 100.00%. Disease prevalence was 80%, 95% confidence interval being 56.34% to 94.27%. Positive predictive value was 100% and negative predictive value was also 100%. When Cohens Kappa value was calculated to analyze the agreement between the observed values of ACHE stain and H&E stains, the number of observed agreements between them were 32 (80.00% of the observations). The Kappa= 0.600 SE of kappa value was 0.126 95% with confidence interval 0.352 to 0.848.The strength of agreement was good according this calculation.

Although the routine H & E and rapid staining methods had an equal sensitivity and specificity, rapid methods score over the routine method with reduced turnaround time The turnaround time was compared between routinely fixed and haematoxylin and eosin stained sections with acetylcholine esterase stain and rapid haematoxylin and eosin stain. The time taken for rapid haematoxylin and eosin was ten minutes. The time taken for acetylcholine esterase stain was about fifty minutes and for routinely stained sections it took about 48 hours (Table 3).

Table 3. Turnaround time noted with ACHE, rapid H&E and routine H&E

Acetylcholine esterase	Rapid H&E	Routine H&E
50 minutes	10 minutes	48 hours

All though rapid haematoxylin and eosin took lesser time than ACH stain, certain patterns that were recognized with the acetylcholine esterase stain were not appreciated with rapid haematoxylin and eosin stain. Two different patterns were observed according to Moore *et al*. (Moore and Johnson, 2005). Different patterns of stained nerve fibers observed with acetylcholine esterase stain with age wise stratification were analyzed. Acetylcholine esterase staining in the nerve plexus between the crypts in lamina propria, submucosa and muscularis mucosa was diagnosed as having pattern A-positive Hirschsprung's disease.

Acetylcholine esterase staining the nerve plexus in the base of the crypts, submucosa and muscularis mucosa was considered as pattern B positive -Hirschsprung's disease. Only one case with equivocal pattern and the hypertrophic nerve bundle was observed, which was also a positive Hirschsprung's disease pattern. Pattern A (9 cases) was more commonly observed than pattern B (6 cases) (Table 4). Aganglionosis could not be assessed perfectly using haematoxylin and eosin stain as ganglion cells normally were scattered and were difficult to find when present in the submucosa.

Table 4. Different patterns observed in ACH stain with age wise stratification

Age	Pattern A	Pattern B	Equivocal with hypertrophic nerves	Negative
1-6months	5	4	1	-
7months – 1 year	-	-	-	1
1-5 years	4	2	-	2
6-12 years	-	-	-	1

And when the hypertrophic nerve twigs formed were very sparse, especially in early infancy, diagnostic difficulty was ensued when H&E was used. Hypertrophic nerve bundles even if it was minimal were highlighted using the acetylcholine esterase, which was difficult when rapid haematoxylin and eosin was used. False positive results were rare, according to the study of Blisard, *et al*. (Blisard and Kleinman, 1986). Particularly, difficulty was encountered in the case of young infants. But H & E clearly complimented the acetylcholine esterase stain. Layer *et al*. in their study had questioned the role of acetylcholine esterase staining if there are clear cut evidence of hypertrophic nerves and ganglion cells (Layer and Kaulich, 1991). But in young infants when hypertrophic nerves are less developed and when the ganglion cells are immature ACH stain clearly compliments.Because the rapid technique diagnoses cases as efficiently as routine stain, the rapid diagnostic method was used as an intra -operative consultation method, thereby waiting period was reduced. As ACHE staining was an on table procedure the results were relayed immediately to the waiting surgeons, facilitating single stage pull through surgery.

DISCUSSION

There are four forms of Hirschsprung's disease.

1. Short segment disease (Classic) - Only a small segment of the rectum and sigmoid colon are involved. Distal sigmoid colon and rectum are affected in 75% to 80% of short segment disease.
2. Ultra short segment disease - Very short segment (<2cm) is involved.
3. Long segment disease - The disease extends beyond the splenic flexure. In 10% of the cases, the disease involves bowel segment proximal to the splenic flexure.
4. Total bowel aganglionosis – In total colonic aganglionosis the disease extends from the anal canal to at least the ileocaecal valve, but does not extend more than 50 cm beyond the ileocaecal valve. The incidence of this form is 5%.Total colonic aganglionosis presents outside the neonatal period, even in the adolescent age and early adulthood.

In Hirschsprung's disease, the main finding is the lack of ganglion cells in the narrow segment of the colon. The aganglionic segment starts distally at the anal ring and extends proximally to a variable length. The internal sphincter has a natural scarcity of ganglion cell close to the anus for a length about 10mm above the dentate line. This is the reason for the general rule to perform biopsies 2-3 cm proximal to the dentate line, according to Teitelbaum *et al*. (Teitelbaum and Coran, 2006)**.**

The affected segment has three zones which include the narrow segment, transition zone and a dilated proximal bowel segment. Histologically, in the narrowed segment there are aganglionosis and hypertrophic nerve bundles. The narrowed segment continues as a transition zone. Transitional zone can be few millimeters to varying centimeters in length. Ganglion cells begin to appear in this zone. Both the presence of hypertrophic nerves and presence of ganglion cells is seen in the transition zone. This continues as a dilated ganglionic segment.

Meier-Ruge did extensive research on the histopathological diagnosis of Hirschsprung's disease and other congenital neuronal intestinal malformations (Meier-Ruge *et al*., 1972). The following criteria for the diagnosis of HSD were formulated by him: Hirschsprung's disease is characterized by aganglionosis in both the Meissner and myenteric plexus. Acetylcholine esterase staining activity is increased in the coarse parasympathetic neurofibrils present in the lamina propria mucosae.

Absence of ganglion cells and the hypertrophic nerve bundles that are present in high numbers is the hallmark of HSD. This is always associated with an increase in acetylcholine esterase in the affected tissue (Qualman *et al*., 1999; Gath *et al*., 2001). Acetylcholine esterase does not stain normally innervated intestinal mucosa (Kamijo *et al*., 1953; Niemi *et al*., 19611 Ariel *et al*., 1983). Histological presence of coarse refractile acetylcholine esterase staining nerve fibrils and the absence of ganglion cells of the inter plexuses should be used in the diagnosis of Hirschsprung's disease according to Meier *et al* (Rouzrokh *et al*., 2010; Barr *et al*., 1985).Garrett *et al* demonstrated that severity with which the case presents and degree of obstruction in Hirschsprung's disease correlated with the acetylcholine esterase stained nerves in the aganglionic zone (Garrett and Howard 1969). The reason behind why acetylcholine esterase levels are raised in Hirschsprung's disease is complex. RET gene, which is the predominant gene identified in Hirschsprung's disease is considered as a potential candidate.

The differential diagnosis includes intestinal neuronal dysplasia. It is a clinical differential for HSD which is diagnosed histologically by the presence of giant submucosal ganglia, containing more than eight nerve cells. Segmental dilatation of the colon, a rare motility disorder affecting children is also a differential for HSD. It can be differentiated from HSD by normally distributed ganglion cells in the colon. Other differentials include internal anal sphincter achalasia in which absence of the rectosphincteric reflex on rectal balloon inflation and the clinical presentation is same as that of Hirschsprung's disease. Histopathologically the presence of ganglion cells on rectal suction biopsy helps to rule out HSD. Hypoganglionosis also a differential is characterized by decreased number of ganglion cells and reduced size of ganglia and wider distances between myenteric ganglia. According to the criteria of Meier-Ruge *et al*, less than 10 ganglion cells/mm (normal -14±3.3) and /or less than 2 ganglions/mm (normal:3.3±1.1) is used to diagnose hypoganglionosis. In contrast to the Hirschsprung's disease, hypoganglionosis do not have nerve plexus hyperplasia. Chronic idiopathic intestinal pseudo-obstruction, slow transit constipation and megacolon are also the mimickers, but can be differentiated histopathologically.

When routine haematoxylin and eosin (H&E) stains are used in rectal suction mucosal biopsies, there are many disadvantages. Submucosa should be equal in thickness to the mucosa in the biopsy specimens. But practically this is not possible. Ganglion

cells are fewer and it is scattered in the submucosa. Hence, evaluation of ganglion cells in the submucosal biopsy using H&E is difficult. This was not in a case when acetylcholine esterase staining was used. The turnaround time was also reduced. This study proved that acetylcholine esterase can be used as a rapid diagnostic tool and it has an advantage over H&E.

Conclusion

Acetylcholine esterase staining, rapid haematoxylin and eosin, and routine formalin fixed sections all had the same diagnostic efficacy. But the turnaround time was very much reduced when rapid diagnostic methods were used when compared with routine formalin fixed sections stained with H&E. Although rapid haematoxylin and eosin method took less time and was less expensive when compared with acetylcholine esterase, certain patterns like aganglionosis and hypertrophic nerve bundles seen using the acetylcholine esterase were not highlighted when rapid haematoxylin and eosin alone was used. Particularly, difficulty was encountered in the case of young infants. Immature ganglion cells are difficult to recognize in frozen sections stained with H&E. But rapid H&E clearly complimented the acetylcholine esterase stain. Intra operative consultation was possible in our setup. This reduced the need for a second surgery, improved the outcome of the surgery and reduced the duration of hospital stay.

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Conflict of interest

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