Original Article

Calretinin versus Hematoxylin and Eosin Stain for Diagnosis of Hirschsprung's Disease; Comparison in Ganglionic, Transitional, and Aganglionic Zones

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Objective: To compare the results of calretinin stain with hematoxylin and eosin (H&E) stain for diagnosis of Hirschsprung's disease.

Materials and Methods: A prospective double-blind diagnostic study was done. Hirschsprung's patients who underwent transanal endorectal pull through (TERPT) surgery between February 2015 and February 2017 were included. The pulled-through specimens were sampled at the ganglionic, transitional, and aganglionic zones. Each specimen was stained with calretinin and H&E. Agreement and kappa analysis were done.

Results: Fifty-one Hirschsprung's patients (153 specimens) were included for analysis. There were 37 males (72.5%) and 14 females (27.5%) with a median age at surgery of four months. Thirty-one specimens showed a negative stain for calretinin (aganglionic bowel) and 33 specimens showed no ganglion cells in the H&E stain. One hundred twenty-two specimens in calretinin stain were consistently positive with ganglionic bowel and 120 specimens in the H&E stain showed ganglion cells. Agreement and Cohen's kappa coefficient were 97.4% and 0.921 (95% confidence interval 0.845 to 0.997), respectively. Disconcordance was found in four specimens. Three out of four were in the transitional zone. One was in the aganglionic zone, which has no muscular layer attached. (Thirty-six specimens had no muscular layer.)

Conclusion: Calretinin stain was found to be comparable with the H&E stain and could be used for diagnosis of Hirschsprung's disease. In rectal suction biopsy specimen in which the muscular layer was not included, both calretinin and H&E can be used.

Keywords: Megacolon, Hematoxylin and eosin (H&E), Calretinin, Rectal suction biopsy (RSB), Full thickness biopsy

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Hirschsprung's disease (HSCR) is defined as congenital absence of parasympathetic ganglion cells in the submucosal and myenteric plexus of the bowel due to failure of migration and differentiation of the neural crest cells to the distal bowel. Formerly, HSCR was called congenital megacolon because aganglionic bowel causes interference with smooth muscle contractility and peristalsis, which leads to colonic dilatation from obstruction. The most common site of the aganglionic segment is below the rectosigmoid colon. Incidence of HSCR is approximately 1:5,000 live-births with male to female ratio around 3 to 4:1[1-3]. Presenting symptoms are neonatal obstruction, chronic constipation, or enterocolitis.

Diagnosis of HSCR comprised of clinical, radiologic, manometric, and pathologic modalities. Clinical symptoms of neonatal distal intestinal obstruction were found in approximately 50% to 90% of cases. Delayed passage of meconium in the first 24 hours was noted in 90% of the cases. Radiologic evaluation usually found with colonic dilatation in plain abdomen and transitional zones with other signs in contrast enema[4]. Anorectal manometry showed an absence of reflex relaxation

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of internal anal sphincter while rectal distension occurred[5]. Rectal biopsy remains the gold standard for diagnosis of HSCR. Histologic findings with hematoxylin and eosin (H&E) stain showing absence of ganglion cells in submucosal and myenteric plexus are the definite diagnosis. Full thickness rectal biopsy clearly provides the investigation of ganglion cells in submucosal and myenteric plexus. However, the procedure is invasive, needs general anesthesia and causes rectal scarring, which could delay the definitive procedure if HSCR was diagnosed[6]. In 1969, rectal suction biopsy (RSB) was firstly introduced by Campbell and Noblett[7]. The procedure was done at the bedside with no sedation required. The obtained specimen contained mucosa, submucosa, and lamina propria. Immunohistochemistry staining was done for diagnosis of HSCR. In a European survey, 39% utilized full thickness rectal biopsy while 61% used RSB to diagnose HSCR[8].

The histological methods and immunochemical stains used are H&E, acetylcholinesterase (AChE), calretinin, S100, nicotinamide adenine dinucleotide-tetrazolium reductase, succinate dehydrogenase, neuron-specific enolase, and lactate dehydrogenase. The primary stains utilized are H&E, AChE, and calretinin. H&E of the submucosa and muscularis propria should be included. Muscularis propria is usually missed in RSB. For AChE, the specimen must be frozen. The process is sometimes complicated.

Calretinin is now used in many institutes. Approximately 30% of the diagnostic markers in RSB are calretinin stained[9]. Calretinin is vitamin D-dependent calcium-binding protein, which binds with calcium in the calcium transport system in the area of neuroexitability. The expression of calretinin in nerve fibrils and ganglion cells in the submucosa and myenteric plexus can be found in the normal colon and small bowel[10]. Therefore, it can be used for HSCR diagnosis even in an RSB specimen in which muscularis propria was not included[11,12].

In Hirschsprung's patients, transanal endorectal pull through (TERPT) was one of the surgical options. Pull through specimens comprised of the aganglionic zone, transitional zone, and ganglionic zone. In the aganglionic pull through segment, only mucosa and submucosa were derived, which was the limitation of H&E staining. In the ganglionic segment, a full thickness of the bowel was obtained where both calretinin and H&E stain could properly be evaluated in[13].

In many countries, RSB is not available. The diagnosis is done by clinical presentation combined with radiologic findings especially contrast enema. The complete diagnostic modalities should combine with the result of histologic evaluation. The histologic staining was one of the barriers in tissue diagnosis. The present study aimed to compare the results of calretinin stain with H&E stain for diagnosis of HSCR and facilitate the use of tissue diagnosis.

Materials and Methods

Study design and participants

A prospective diagnostic study included patients diagnosed with HSCR that underwent TERPT surgery with or without abdominal assistance. The Ethical Committee of Faculty of Medicine, Chiang Mai University approved this study. Consecutive Hirschsprung's patients attending the faculty of Medicine, Chiangmai University, Thailand, between February 2015 and February 2017 were invited to participate in this study. HSCR was diagnosed by clinical presentations and imaging (transitional zone was seen in barium enema) or rectal biopsy. The pull through specimens were biopsied in three zones, aganglionic, transitional, and ganglionic. The sequence of the three zones were randomly sent for pathologist. Central randomization was done in the process of allocation concealment. The specimen allocation was known when the patient entered the operative theater by telephone or text message from the research coordinator. The pathologist and data analyst were blinded. The data about gender, age at operation, operative methods, length of the resected specimens, and levels of transitional zone were collected.

Test methods

Specimen collections: The pull through specimens from the patients that underwent surgery were identified into three zones, the aganglionic zone, the transitional zone, and the ganglionic zone. A 1×1-centimeter piece of full thickness biopsy in each zone was taken. The aganglionic zone specimen was cut at the point of one centimeter above the end of the specimen (distal side). The transitional zone specimen was cut at the point that the bowel specimen changed caliber. The top of the specimen was cut and sent as the ganglionic zone. Each specimen was stained with calretinin and H&E to perform a matched pair comparison of the results of the stain (Figure 1). The specimens were fixed in 10% formalin and randomly labelled A, B, and C for blinding the pathologist and technician.

Histopathological and immunohistochemistry

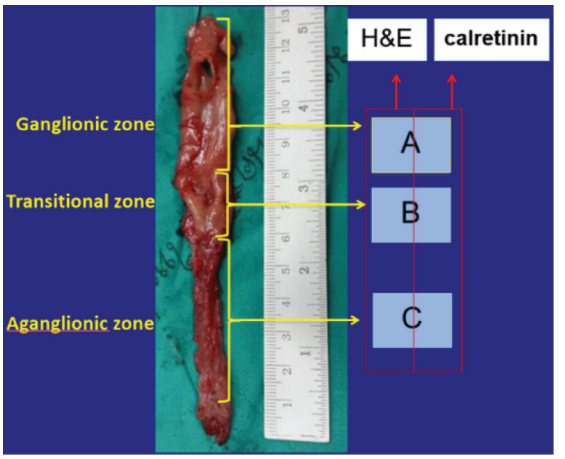

Figure 1. Specimen collection method.

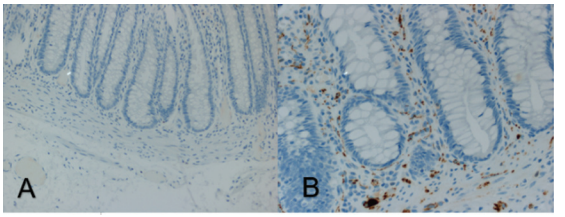

Figure 2. Calretinin staining; (A) aganglionic bowel, (B) ganglionic bowel.

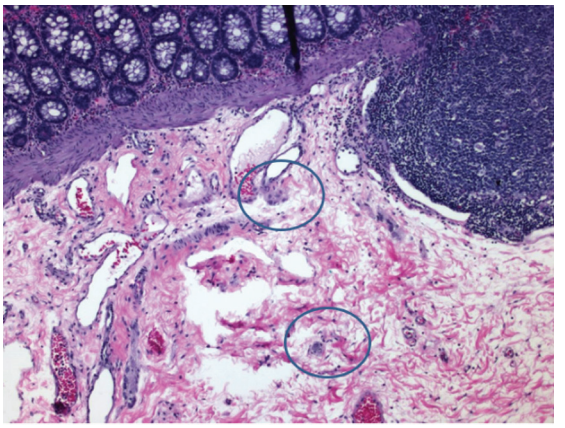

Figure 3. H&E staining; ganglion cells in circle.

analysis: The tissue samples had been fixed routinely in 10% neutral buffered formalin and embedded in paraffin. Four micrometer sections were cut, and histology was verified by H&E staining. Immunohistochemical staining was carried out for calretinin on representative four µm sections cut from a formalin-fixed paraffin embedded block. An immunohistochemical study was prepared using a Benchmark XT, automated immunostainer from Ventana Medical System. The authors' institute used ready-to-use (RTU) antibodies, based on DAK-Calret1, Dako.

In calretinin immunohistochemical stain, positive stain was defined as a stain that had highlighted ganglion cells, nerve trunks, nerve fibrils of the superficial submucosa, muscularis mucosae, or lamina propria. Negative stain was defined as a stain that had no highlighted ganglion cells, nerve trunks, nerve fibrils of the superficial submucosa, muscularis mucosae, or lamina propria[14]. Positive stain was consistent with ganglionic bowel and negative stain was consistent with aganglionic bowel (Figure 2).

In H&E stain, positive stain was defined as a stain that had highlighted ganglion cell in the submucosa, muscularis mucosae, and lamina propria. Negative stain was defined as a stain that had no highlight ganglion cells in the submucosa, muscularis mucosae, and lamina propria. Positive staining was consistent with non-HSCR and negative staining was consistent with HSCR (Figure 3). The authors used H&E stain as a standard reference in the present study. Both calretinin and H&E were tested in each specimen.

Statistical analysis

Statistical analysis was performed with commercial statistical software, Stata Statistical Software, version 15.1 (StataCorp LLC, College Station, TX, USA). Clinical data regarding gender, age at operation, length of resected specimen, and level of transitional zone were analyzed with descriptive statistics and frequencies. Sample size calculation based on non-inferiority trial for binary data was done at 5% significant level and power of 80%. The proportion of true positive of calretinin and H&E from a study of Kannaiyan et al were 0.81 and 0.53, which gave a sample size of 23 per test[15]. Sensitivity, specificity, accuracy, agreement, and Cohen's kappa concordance coefficient were used to compare the results of the two staining techniques.

Results

During the present study period, fifty-one patients diagnosed with HSCR and that underwent TERPT were included in the study. Thirty-seven patients were male (72.5%) and 14 patients were female (27.5%). The median age at time of surgery was four months. Most transitional zone was located at the rectosigmoid colon. Characteristics of the patients are shown in Table 1.

One hundred fifty-three specimens from 51

Table 1. Characteristics of HSCR patients (n=51)

Characteristics	n (%)
Sex	
Male	37 (72.5)
Female	14 (27.5)
Age at operation (months); median (min-max)	4 (1 to 56)
Length of resected specimen (cm); mean±SD	15.8±7.2
Transitional zone	
Short	17 (33.3)
Rectosigmoid	23 (45.1)
Long	4 (7.8)
Unknown due to previous ostomy	7 (13.7)

SD=standard deviation

Table 2. Comparison between the results of H&E and calretinin staining (n=153)

Histological staining	H&E; n (%)		Total
	No ganglion cell	Presence of ganglion cell	
Calretinin			
Negative staining	30 (96.8)	1 (3.2)	31
Positive staining	3 (2.5)	119 (97.5)	122
Total	33	120	153

H&E=hematoxylin and eosin

Aganglionic bowel; H&E=no ganglion cell, calretinin=negative staining

Ganglionic bowel; H&E=presence of ganglion cell, calretinin=positive staining

patients were obtained. The results of H&E and calretinin staining are reported in Table 2. Thirty-one specimens showed negative stain of calretinin (aganglionic bowel) and 33 specimens showed no ganglion cells in the H&E stain. One hundred twenty-two specimens in calretinin stain were positive, which was consistent with ganglionic bowel and 120 specimens in H&E stain showed ganglion cells.

Disconcordance results between H&E and calretinin occurred in four specimens. Three had results in the transitional zone and the other was in the aganglionic zone. One specimen had ganglion cells in a negative calretinin stain. Three had absence of ganglion cells in positive calretinin result. No disconcordance was found in the ganglionic zone. Distribution of the disconcordance of the staining results by layers of bowel is shown in Table 3.

Agreement and Cohen's kappa concordance coefficient between calretinin and H&E staining were done. The authors found that agreement was 97.4%

Table 3. Distribution of the disconcordance of the staining results by layers of bowel

Disconcordance specimens/zone	Calretinin; n (%)		H&E; n (%)	
	Submucosal layer	Muscular layer	Submucosal layer	Muscular layer
1/transitional	Positive	Positive	No ganglion	No ganglion
2/aganglionic	Positive	N/A	No ganglion	NA
3/transitional	Negative	Positive	No ganglion	No ganglion
4/transitional	Negative	Negative	Ganglion	No ganglion

H&E=hematoxylin and eosin; N/A=not applicable due to no muscular layer attached

Table 4. Sensitivity, specificity, accuracy, agreement, and kappa analysis of calretinin by using H&E as standard reference

Parameter	%	95% confidence interval
Sensitivity	90.9	86.4 to 95.5
Specificity	99.2	97.7 to 100
Accuracy	97.4	-
Agreement	97.39	-
Kappa analysis	0.921	0.845 to 0.997

and Cohen's kappa concordance coefficient was 0.921 (95% confidence interval 0.845 to 0.997). Sensitivity, specificity, accuracy, agreement, and kappa analysis of calretinin by using H&E as standard reference are shown in Table 4.

Discussion

HSCR is a complex developmental gastrointestinal disorder characterized by the absence of ganglion cell in the intestine and hypertrophic nerve fibers. Neonatal abdominal distension, delayed passage of meconium, or constipation in infants may be the presentations. Various diseases mimic the symptoms of HSCR such as infantile dyschezia, lactase deficiency, and cow milk protein allergy[16,17]. Even radiologic diagnosis might imitate HSCR[18]. Tissue diagnosis is quite important.

The classic diagnosis of HSCR is a full thickness rectal biopsy with H&E stain showing absence of ganglion cells in the submucosa and muscularis propria. However, patients who underwent rectal biopsy performed under general anesthesia presented with rectal scar at the time of the full surgery. In present time, RSB is a method to exclude the HSCR by demonstrating the presence of submucosal

ganglion cells. RSB can be performed without general anesthesia. The procedure can be done with various staining techniques for diagnosis of Hirschsprung disease. In 2016, Friedmacher and Puri conducted an international survey in the pattern of RSB and staining methods. The consensus is still not settled[9].

There are various histological markers and stains for rectal mucosal biopsy in HSCR. If all the layers of the bowel were included, H&E would be the preferred method. Most studies used histopathology staining by H&E as a standard reference[19]. Some studies used AchE essay for a standard reference[13]. Some standard references were tailor-made with the clinical diagnosis[14]. The present studies used calretinin and H&E as the diagnostic module in the pathologic specimen. H&E was a standard reference. These two tests were previously known as good diagnostic value modules. H&E is available, has simple specimen preparation, and a clear interpretation process. However, the limitation is as previously stated, that all the layers of the bowel should be included. Calretinin is another method that is easy to use. This immunohistochemical stain is used in many pathological centers. Specimen preparation is almost the same as H&E. The interpretation technique is also not complicated and easy to learn. AchE assay is not available in many centers. The process requires high cost and specimen must be frozen before the process. Therefore, the authors used calretinin and H&E as diagnostic tests for rectal specimens.

RSB system is not available in many areas. It had been introduced to the authors' hospital in 2017. Previously, the diagnosis of HSCR was done by clinical and radiologic evaluation. One of the authors' limitation was overdiagnosis of HSCR. Some of the specimens in the aganglionic zone were found present of ganglion cells.

From the present study, three out of four specimens that had different results between calretinin and H&E, were found in the transitional zone. These findings might occur due to the unpredicted distribution of ganglion cells in this zone. In 2018, Najjar et al reported an increased number of calretinin staining in the mucosa in the transitional zone[20]. This finding supported that in areas where there were small numbers of ganglion cells, calretinin might show positive results. One specimen in the aganglionic zone demonstrated a positive result in calretinin staining and negative for ganglion cells in H&E. The interpretation might be difficult for H&E in this specimen zone due to no muscular layers involved but should be considered as an over interpretation of the positive calretinin results. Therefore, the authors recommend using the combination of calretinin and H&E to the diagnosis by increasing the accuracy of Hirschsprung diagnosis. In cases of disconcordant results, clinical follow up or repeat biopsies are very useful.

Nowadays, calretinin is used as an additional tool for diagnosis of HSCR and can be done with RSB. Tran et al conducted a prospective study and reported that calretinin had a high diagnostic accuracy and should be considered as the primary method for the diagnosis of HSCR with specificity of 99.1%, sensitivity of 100%, positive predictive value of 98.8%, negative predictive value of 100%, and Cohen's kappa index of 98.9%(14). Similarly, de Haro Jorge et al reported a sensitivity of 97.5% and specificity of 97.1%[21]. The present study was a prospective diagnosis study. The authors confirmed that calretinin can be used for diagnosis of HSCR with sensitivity, specificity, agreement, and Cohen's kappa concordance coefficient between calretinin and H&E staining of 90.9%, 99.2%, 97.4%, and 0.921 (95% confidence interval 0.845 to 0.997), respectively. In addition, the authors also confirmed the usage of calretinin in rectal biopsy specimen with no muscular wall that simulated the RSB specimen and comparable to H&E in all three zones of the present study.

Comparisons of calretinin and AchE activity stains were reported in many previous studies[19,22,23]. Most of the results showed that calretinin was superior in diagnosis of HSCR. In the present study, the authors did not use AchE assay because of unavailability. Furthermore, many studies reported additional stains for diagnosis of HSCR. Jiang et al reported that staining of nerve fibers for S100 and PGP9.5 showed submucosal neural hypertrophy on S100 staining and PGP9.5 staining[11]. However, there is no strong evidence supporting that S100 and PGP9.5 stains are better than calretinin or H&E stains.

HSCR can be practically diagnosed by clinical and contrast enema. The presence of transitional zone in the contrast enemas assumed the diagnosis of Hirschsprung disease. Tissue diagnosis warrant the diagnosis of Hirschsprung disease. The present study was conducted with the specimen only. Further studies combining clinical, radiologic, and tissue diagnosis should be done.

Conclusion

Calretinin stain was comparable with H&E stain and could be used for diagnosis of HSCR with high sensitivity, specificity, and accuracy. In RSB specimen

that does not include muscular layer, both calretinin and H&E in combination could be used.

What is already known in this topic?

The immunohistochemistry stains help in diagnosis of Hirschsprung disease. There are many special stains used for diagnosis. Standard reference is still using H&E stain. Calretinin is already known to be a very useful stain for diagnosis of HSCR.

What this study adds?

Calretinin stain can be used in specimens without muscular layer, which is the weak point of H&E stain. In RSB specimen, calretinin is helpful. This immunohistochemical stain can be used for detection of ganglion and nerve fiber in the ganglionic zone, transitional zone, and aganglionic zone.

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Conflicts of interest

The authors declare no conflict of interest.

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