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Diagnostic value of rectal suction biopsies using calretinin immunohistochemical staining in Hirschsprung's disease

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ABSTRACT

Background: The study investigates the diagnostic value of calretinin immunohistochemical staining (CIS) on rectal suction biopsies (RSB) in Hirschsprung's disease (HD).
Methods: A prospective study was conducted at Children's Hospital 2 in Ho Chi Minh City, Vietnam, from January through December 2015. Patients suspected of HD during this period underwent RSB and were followed in order to assess the accuracy of the diagnostic test with CIS compared with conventional histology (H&E).
Results: A total of 188 children with RSB were investigated. Median age was 7.1 (range 0.2–159) months with 65.4% boys. HD was confirmed in 80 (42.6%) children. There were 1 false positive and no false-negative cases. The sensitivity and specificity were 100% (80/80) and 99.1% (107/108) for CIS and 100% and 85.2% for H&E, respectively. Cohen's kappa coefficient was 0.9891 with a diagnostic accuracy of 99.5% for CIS, compared with 0.8303 and 91.5% for H&E, respectively. There were no serious complications related to the RSB.
Conclusion: RSB with CIS is a useful diagnostic method for HD, with easy interpretation and no need for cryostat. CIS has a high diagnostic accuracy and should be considered as the primary method for the diagnosis of HD by RSB.
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Hirschsprung's disease (HD) is a common cause of large-bowel obstruction in infants. The prevalence is about 1 per 5000 live births, with an estimated prevalence of 1.4 per 5000 live births in Asian populations [1]. The HD diagnosis is based on clinical symptoms, contrast enema, anorectal manometry and histologic findings. Despite many advances in the management of HD in recent years, the clinical diagnosis is still challenging in a significant number of cases. For this reason rectal biopsy with histopathology is considered essential before surgery [2].

In 1969, Campbell and Noblett were the first to perform transanal rectal suction biopsies (RSB) with a special tool in order to facilitate the diagnosis of HD [3]. RSB has many advantages over full thickness biopsy, including the absence of general anesthesia, less trauma and ease of performance. Currently RSB is the most used sampling technique for HD. Conventional hematoxylin and eosin (H&E) staining combined with acetyl cholinesterase (AChE) staining on RSB has been, until now, the standard method for primary histological diagnosis of HD [4].

In 2004, Barshack et al. demonstrated that there is no reactivity for calretinin immunohistochemical staining (CIS) within aganglionic colon in HD [5]. Calretinin is a calcium-binding protein involved in calcium signaling. The presence of ganglion cells consistently correlates with positive CIS reactivity of thin nerve fibrils in the lamina propria, muscularis mucosa and superficial submucosa [6]. Subsequently, some authors have shown that CIS is equivalent or even better than other diagnostic tests for HD. CIS has been raised as a potential substitute for AChE [7–15]; however, there are very few prospective studies of the use of CIS in HD [11,16].

In Vietnam, there have been no reported data about RSB for the diagnosis of HD. For these reasons we conducted a study in an attempt to investigate the results of RSB with CIS to diagnose HD, compared with conventional histology (H&E). The primary end point of the study was to assess the values of the histologic diagnostic tests. The second aim of the study was to evaluate the safety and efficacy of RSB in Vietnam.

1. Materials and methods

A prospective study was conducted at Children's Hospital 2 in Ho Chi Minh City, Vietnam from January through December 2015 in accordance

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with the research ethics committee of the hospital. All patients suspected of HD underwent RSB. The samples were sent to the Department of Pathology, University of Medicine and Pharmacy, Ho Chi Minh City for full analysis. The specimens were divided into 2 parts, stained for H&E and CIS, and interpreted by a single senior pathologist. Patients were excluded if they had previously undergone surgery for HD.

A diagnosis of HD was made by considering RSB histologic analysis, as well as clinical features, radiologic findings, and anorectal manometry if available. A surgical full thickness biopsy (according to the Swenson technique) was performed after RSB if the tissue was insufficient for analysis with H&E staining or when there were clinical inconsistences between the different diagnostic modalities. Patients were then managed by either a definitive surgical treatment in cases of HD or follow-up with appropriate treatment in cases of non-HD. Inclusion criteria into the study were patients suspected for HD who were submitted to RSB and in whom final diagnosis was obtained on the surgical specimen (in case of positive diagnosis of HD) and those who underwent follow-up for 6 months after RSB (in case of negative diagnosis of HD).

1.1. Rectal suction biopsy technique

Study patients did not need general anesthesia. Premedication was provided in non-cooperative or anxious children. The biopsy instrument was Medtronic® Scheye's forceps with a suction hole size of 3 mm. Three samples were taken consecutively in 3 positions: 3 o'clock, 6 o'clock, and 9 o'clock, at a distance of about 4 cm above the anal margin [17].

1.2. Staining techniques

After RSB, the specimens were fixed in 10% neutral buffered formalin solution. Staining was performed on paraffin wax-embedded 3–4 μm thick sections. Each sample was cut into serial sections of 20–50 slices: 5 slices were used for CIS; all other slices were stained for H&E staining.

1.2.1. Calretinin immunohistochemistry

CIS was performed with the Ventana Benchmark XT® automatic machine. Cell conditioning solution 1 was used to identify antigen. Calretinin antibody (SP65) Rabbit monoclonal Primary Antibody, Lot F00109, was used for incubation. Normal colon tissue was routinely used as the control for all CIS.

The results of CIS were considered as: (1) incompatible with HD when showing positive reactivity for calretinin immunohistochemical staining within the ganglion cells and/or thin nerve fibrils in the lamina propria, muscularis mucosa and superficial submucosa; (2) compatible with HD when showing no reactivity for calretinin immunohistochemistry.

1.3. Hematoxylin & eosin staining

The results of H&E were considered as: (1) incompatible with HD when showing the presence of ganglion cells on the slices of the samples; (2) compatible with HD when showing no ganglion cells on the slices within at least 2 samples containing adequate submucosal tissue; (3) inconclusive when having fewer than 2 samples with adequate submucosal tissue for analysis, in which case repeat RSB was performed.

1.4. Statistics

Data were reported as mean and standard deviation (SD) or median and range for continuous variables, according to their distribution, and as number and proportion for categorical variables. The chi-square test or the Fisher exact test was used to compare proportions. The diagnostic tests were evaluated for sensitivity, specificity, positive predictive value, and negative predictive value. Cohen's Kappa concordance coefficient was used to evaluate the accuracy of the tests [18]. IBM SPSS Statistics version 20 (SPSS Chicago, IL) was used for statistical analysis.

2. Results

2.1. Patients characteristics

A total of 188 patients suspected of HD underwent RSB. Patients characteristics are illustrated in Table 1. Of note, 135/188 (71.8%) of patients were less than one year of age and 21/188 (11.2%) were neonates. The youngest patient who underwent RSB was 5 days old. The lowest weight was 2.1 kg. In 6/188 (3.2%), the RSB had to be repeated because insufficient tissue was obtained.

2.2. Histopathology

The results of histologic findings (H&E staining, CIS and final histology) of all the patients suspected of HD in our study are figured out in Table 2.

2.2.1. Hematoxylin & eosin staining

Ganglion cells were identified on RSB in 92 patients on H&E stained slides; those cases were considered as incompatible with HD. In 96 patients, ganglion cells were not demonstrated on the H&E stained slides (in all 3 specimens); those cases were compatible with HD.

2.2.2. Calretinin immunohistochemistry

Calretinin immunohistochemistry was positively stained in 107 patients; those cases were considered as incompatible with HD (Fig. 1A, B, C). Absent reactivity for CIS was seen in 81 cases; those cases were considered as compatible with HD (Fig. 1D). The latter group included 3 special cases (2 with slight positive CIS within thick trunk nerve fibers (Fig. 2C, D) [9,19] and 1 with positive reactivity only in mast cells) (Fig. 2A) [9].

2.3. Final colectomy analysis

During the time of the study, 81 patients underwent operation for HD. All the resected segments were fully evaluated to determine not only the final diagnosis but also the extent of aganglionosis. Histologic analysis of the resected segments confirmed HD in 80 cases, but showed normal innervation in 1 case. All 81 cases showed no reactivity of CIS on

Table 1
Patients characteristics, N = 188.

Demographic characteristics		Results
Age (months)		
Mean (SD)		11.8 (20.2)
Median (range)		7.1 (0.2–159.3)
Weight (kg)		
Mean (SD)		7.2 (6.6)
Median (range)		4.5 (2.1–35.0)
Sex, n (%)		
Male		123 (65.4)
Female		65 (34.6)
Clinical characteristics	Diagnosis	
	Non Hirschsprung (N = 108) N (%)	Hirschsprung (N = 80) N (%)
Age group		
<30 days	10 (9.2)	11 (13.8)
1–12 months	60 (55.6)	54 (67.4)
12–36 months	19 (17.6)	13 (16.3)
>36 months	19 (17.6)	2 (2.5)
Delayed meconium >24 h		
Yes	46 (42.6)	66 (82.5)
No	48 (44.4)	12 (15.0)
Unclear	14 (13.0)	2 (2.5)
Diagnosis at RSB		
Constipation	67 (62.0)	62 (77.5)
Neonatal obstruction	32 (29.6)	14 (17.5)
Intestinal perforation	7 (6.5)	3 (3.7)
Severe enteritis	2 (1.9)	1 (1.3)

Table 2
Diagnostic test with hematoxylin & eosin staining and calretinin immunohistochemical staining in 188 patients.

Histologic staining	Diagnosis		
	Hirschsprung	Non Hirschsprung	Total
Hematoxylin & eosin*			
Absence of ganglion cells	80	16	96
Presence of ganglion cells	0	92	92
Calretinin immunohistochemistry†			
Absence of reactivity of CIS	80	1	81
Presence of reactivity of CIS	0	107	107
Final histology	80	108	188

* Specificity 92/108 (85.2%); sensitivity 100% (80/80); positive predictive value 80/96 (83.3%); negative predictive value 92/92 (100%); accuracy 172/188 (91.5%); Cohen's kappa index 83%.

† Specificity 107/108 (99.1%); sensitivity 100% (80/80); positive predictive value 80/81 (98.8%); negative predictive value 107/107 (100%); accuracy 187/188 (99.5%); Cohen's kappa index 98.9%.

RSB and no ganglion cells on analysis of H&E stained. There is, therefore, 1 false positive (positive diagnosis for HD based on RSB histologic diagnosis, but no HD at resection) (Table 2).

Concerning the length of the aganglionic segment, full analysis of the colectomy segments in 80 cases with a diagnosis of HD showed the following: 11 patients with ultrashort disease, 48 patients with rectosigmoid disease, 9 patients with transverse and descending colon disease, 3 patients with right colon disease, and 9 patients with a total or near-total colon disease.

2.4. Accordance between calretinin immunohistochemistry and H&E staining

Whereas 81 cases were diagnosed with HD by showing no reactivity of CIS as shown in Fig. 1B, 96 cases showed no ganglion cells on H&E stained slides. H&E staining and CIS therefore did not correlate in 15 cases. In these 15 cases, CIS reactivity was positive within fine nerve fibrils, but ganglion cells were not found on H&E stained slides. Full thickness rectal biopsies were performed in these 15 cases, and ganglion cells were detected in all specimens, correlating with the CIS staining results.

In the remaining 92 cases, HD was simultaneously excluded by the presence of ganglion cells on H&E staining and by the positive patterns of CIS reactivity.

2.5. Complications of RSB

There were no serious complications following RSB (including perforation, pelvis sepsis, internal organ damage or massive hemorrhage). Four patients presented with bleeding immediately after RSB, requiring transanal sutures. Most other patients presented with slight bleeding that stopped spontaneously.

3. Discussion

HD is a common disorder in pediatric surgery, especially in Asian populations [1]. In Vietnam and other developing countries the diagnosis of HD is often made based on clinical and radiologic findings; however, correlation between contrast enema findings and histological transition zone remains low [20]. Histological diagnosis by AChE staining is not readily available in developing countries because this technique requires fresh tissue staining, expensive technology and experienced pathologists [6–8]. In contrast, CIS is much easier to perform and interpret [7].

In terms of diagnostic accuracy, our study showed that the sensitivity and specificity of CIS were very high (99.1% and 100%, respectively), which is comparable to results of other authors [11,19,21,22]. In comparison, a systematic review with meta-analysis by Friedmacher et al.

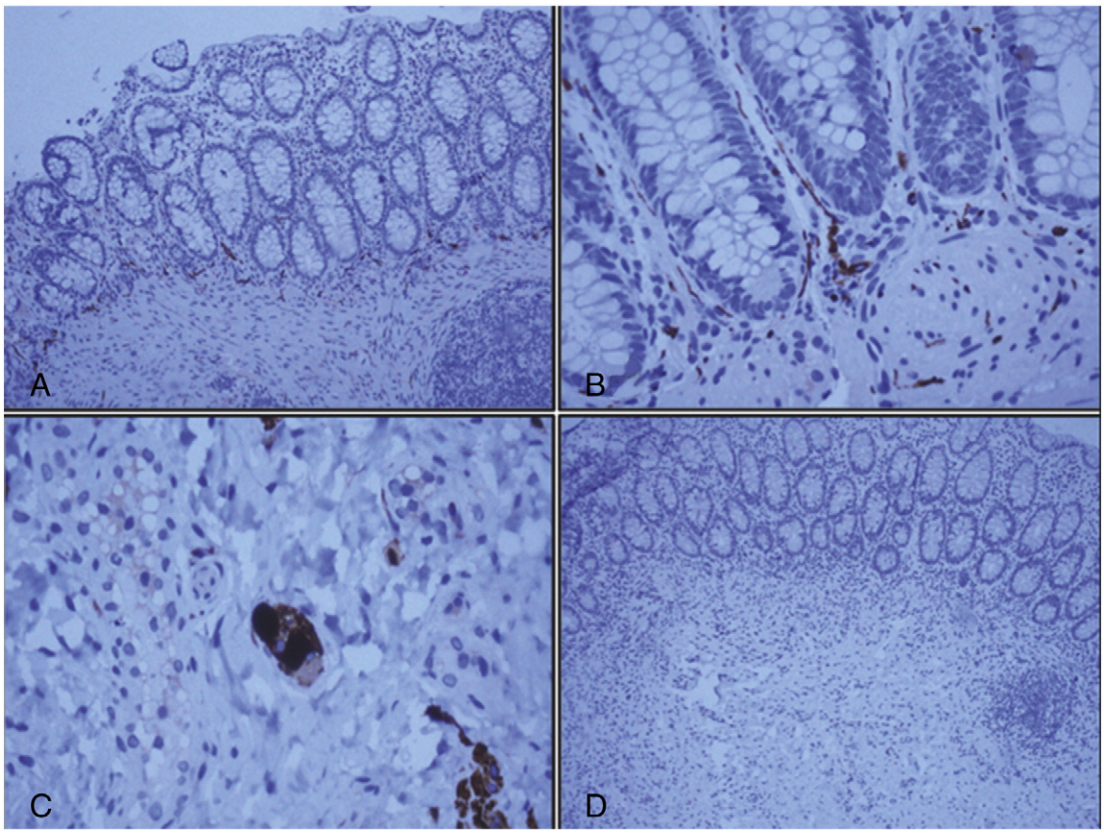

Fig. 1. Patterns of calretinin immunohistochemical staining (CIS) on rectal suction biopsies. Presence for CIS reactivity in fine nerve fibrils, easily detectable within the submucosa and the muscularis mucosa (Fig.1A, ×10) and/or sometimes in the lamina propria (Fig.1B, ×40). Fig.1C, ×40, shows clearly the presence of CIS reactivity in ganglion cells in the submucosa. These patterns exclude the diagnosis of Hirschsprung. Fig.1D, ×10: total absence of CIS reactivity within the submucosa, the muscularis mucosa, and the lamina propria. This pattern is compatible with Hirschsprung's disease.

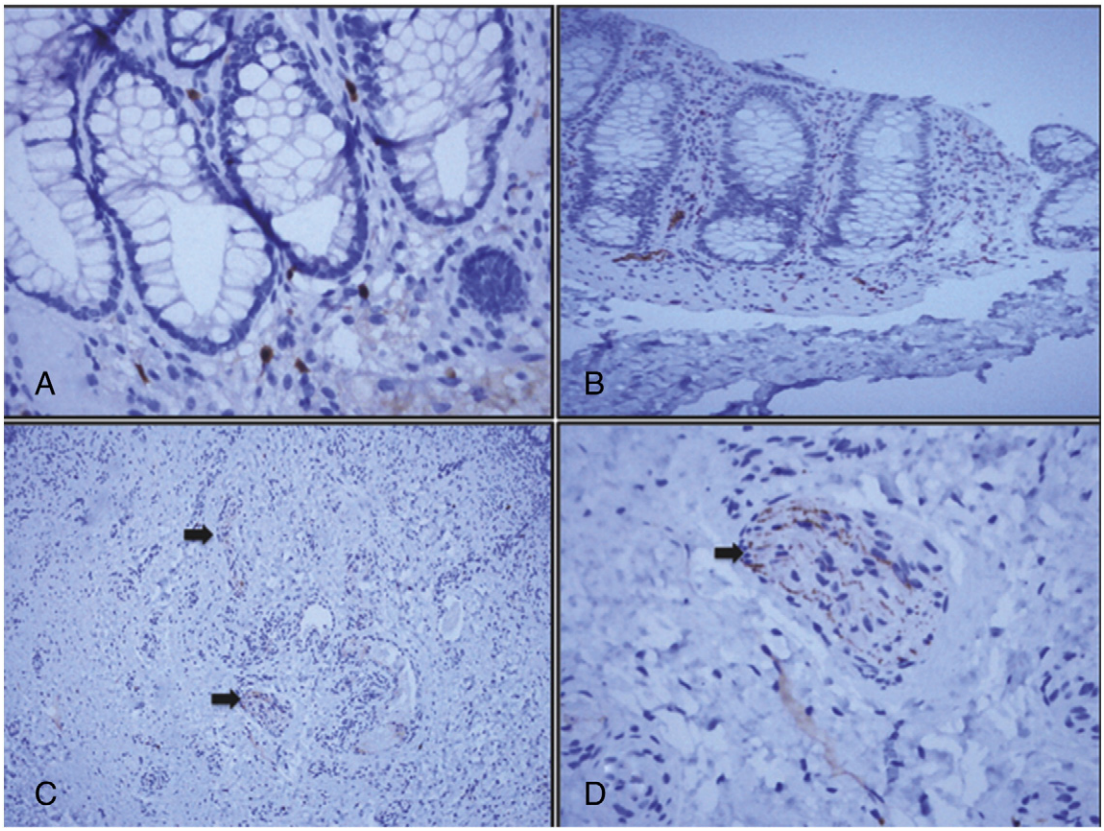

Fig. 2. Pitfalls in the analysis of calretinin immunohistochemical staining (CIS) on rectal suction biopsies. Fig. 2A, ×40: presence of CIS reactivity within mast cells in the submucosa, the muscularis mucosa and the lamina propria in a patient with a history of long-term constipation and enteritis; the diagnosis of Hirschsprung's disease was confirmed (operated at the age of 3 years). Fig. 2B, ×10: presence of CIS reactivity with inadequate tissue for H&E analysis (inconclusive on H&E stained slices). Fig. 2C, ×20 and 2D, ×40: reactivity of CIS within hypertrophied extrinsic nerve fibers within the submucosa (black arrows). The diagnosis of Hirschsprung's disease was confirmed with ultrashort form (aganglionosis limited to 3–4 cm above pectinate line).

showed that the mean sensitivity of AChE staining was 96.8% and the mean specificity was 99.4% [4]. Further, de Arruda Lourencao et al. showed that CIS was comparable or even better than AChE staining for diagnostic accuracy in a prospective study including 43 patients [11]. Our prospective cohort study included 188 patients and confirmed the previous published results on a much larger population of patients.

When compared with conventional H&E staining, our study demonstrates that sensitivity of CIS and H&E staining were equal at 100% (80/80); however, specificity of CIS was significantly higher than H&E (99.1% [107/108] for CIS compared to 85.2% [92/108] for H&E, $p < 0.001$). In addition, Cohen's kappa score of CIS was 0.9891, compared to 0.8303 for H&E.

In our study, 15 false positives were found if using only H&E staining; HD was excluded in all these patients using CIS. With clinical follow-up for 6 months of patients with positive CIS only, thus far no false-negative cases have been identified. In the literature, in cases where the standard technique (both AChE and H&E staining) was used, the percentage of cases with inconclusive or equivocal results is not negligible [7,11]. Our study therefore supports the use of CIS as the primary method for the diagnosis of HD.

In our study, there was 1 false positive using CIS. This could be explained by RSB specimens taken from the physiologic aganglionic zone, because of a technical error. To avoid this error it is recommended to perform biopsies 2 to 3 cm above the pectinate line [11], and our practice is to perform RSB in 3 directions at a distance of 4 cm above the anal margin. While some pathologists recommend doing rectal biopsies at different levels, we felt it would be difficult and dangerous to do so. Since the median weight of the patients in our study was 4.5 kg (range from 2.1 to 35.0), we felt it would be unsafe to perform deep RSB on infants for fear that bleeding that could not be controlled.

In the literature, AChE staining has suboptimal results in certain cases, and CIS offers some potential advantages over AChE staining. In patients with total colonic aganglionosis or prematurity, nerve hyperplasia may not be seen using AChE staining [23]. One explanation is that AChE staining is a reaction secondary to aganglionosis, and it takes time to accumulate AChE enzymes in extrinsic hypertrophied nerve fibers. If RSB is performed too early in the neonatal period false-negatives are possible [23], and repeated biopsies are often required to obtain a diagnosis of HD. In contrast, in our study we were able to make a diagnosis of HD using CIS in 21/21 (100%) of neonates, even when RSB was performed within a few days of birth. In 7 cases of prematurity and low birth weight CIS gave an accurate diagnosis of HD. In cases of long segment forms of aganglionosis, AChE staining may also provide false-negative results. In these forms, the extramural parasympathetic innervation is constantly associated with moderate hypoplasia perturbing AChE staining [24]. In our series, 12/80 patients had total or near-total colon HD, and an accurate diagnosis was made in all patients by using CIS.

Another advantage of CIS is that a diagnosis can be made even when there is inadequate tissue on RSB for diagnosis by H&E and AChE staining (Fig. 2B) [25]. In 6 of our cases a thin sample of submucosa was obtained, which was inadequate for analysis with H&E staining. Using CIS for these cases, 4 cases of HD and 2 cases of non-HD were successfully diagnosed. Results were confirmed after H&E staining on full thickness biopsies. The presence of ganglion cells is strongly correlated with a positive reactivity of CIS in thin nerve fibrils within muscularis mucosa or even in lamina propria [7,10,11].

According to our study design, any patient with inadequate tissue for H&E staining underwent surgical full thickness biopsy to confirm the diagnosis. Because of the results of our study we changed our practice and now clinically follow patients whose RSB show CIS positivity within fine nerve fibrils. We repeat rectal biopsies only in patients with persistent

constipation or any other issue suggesting HD, avoiding repeat biopsies in many patients. In the 6 months' follow-up in this study, we repeated full thickness rectal biopsy in 5 patients because of either persistent severe constipation (2 patients) or physiologic obstruction after closing a transient enterostomy in the neonatal period (3 patients). These cases could be related to intestinal neuronal dysplasia or immature ganglion cells because repeat rectal biopsies and operative samples all excluded the diagnosis of HD by demonstrating the presence of ganglion cells [26].

As compared with standard method (H&E combined with AChE staining), the interpretation of CIS is much easier and does not require expert pathological interpretation; response is a categorical variable (either "yes or no" or "positive or negative") (Fig. 1) [7]. On the contrary, AChE staining requires an expert for interpretation in many cases. Fewer slices and less tissue are needed for CIS analysis [23]. In fact, in our study we used only 5 slices for CIS compared to 20–50 slices for H&E staining of each sample. In the literature, CIS has been shown to be a less time-consuming and cost-effective method [12].

Ultrashort HD is also difficult to diagnose. Contrast enema is usually inconclusive in these cases. In our series 11/80 patients had ultrashort HD after full analysis of the colectomy specimens. Remarkably, 2 of our specimens had light positive CIS reactivity in some thick nerve trunks, all others patterns for CIS reactivity being totally absent (Fig. 2C, D). This phenomenon has already been described by Guinard-Samuel et al. and could lead to the prediction of a short form of HD in these situations, restricted to rectal and sigmoid colon [19]. Indeed, slightly positive staining could be because of the fact that RSB reached the transitional zone just above the short segment of rectal aganglionosis. This observation needs further histologic studies on a larger number of cases.

In patients with a long history of persistent constipation and recurrent enteritis, the histological diagnosis of HD is also challenging. In these cases, inflammatory mast cells are typically stained with CIS [9]. In our series one such patient presented at 3 years of age with a massively dilated rectum and a history of recurrent enteritis and constipation. CIS on RSB was positive only in mast cells. After colonic resection the operative segment showed HD on H&E staining.

Similar to H&E staining where the presence of ganglion cells proves the absence of HD, one criticism of CIS is that positive staining demonstrates an absence of disease. In contrast, positive AChE staining proves the presence of disease.

To our knowledge, this is the largest prospective study of RSB using CIS in the literature. And for the first time in Vietnam RSB was performed in order to confirm the diagnosis of HD before operation. Developing histological techniques that are reliable, easy to interpret, inexpensive, and highly sensitive and specific is critically important not only in developing countries but elsewhere as well. Our study shows that CIS could become the new gold standard for histological diagnosis of HD, even in very difficult cases. One shortcoming of this study is the inability to compare CIS results on RSB with AChE staining, which was not possible because the required materials for AChE staining were not available at our institution.

4. Conclusions

This prospective cohort study shows that CIS after RSB is a safe, simple, and reliable method for the diagnosis of HD. With very high diagnostic accuracy, comparable or even better than AChE staining, CIS should be considered as the preferred technique for the diagnosis of HD on RSB. CIS is superior to AChE staining in neonates or long and ultra-short segment HD. CIS is inexpensive and easy to interpret, making this technique useful in resource-poor settings, but CIS might prove to be the technique of choice for interpretation of RSB in high-income countries as well.

Conflict of interest

The authors declare that they have no competing interests.

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