Original Article

Diagnostic Roles of Calretinin in Hirschsprung Disease: A Comparison to Neuron-Specific Enolase

Zaidoon A. Musa, Ban J. Qasim[1], Haider F. Ghazi[2], A. Wahab A. K. Al Shaikhly[3]

Specialist Pathologist Physician, Al-Emamain Al-Kadhimain Medical City, Departments of [1]Pathology and Forensic Medicine and [2]Microbiology, College of Medicine, Al-Nahrain University, [3]Department of Pharmacy, Al Rasheed University College, Baghdad, Iraq

Address for correspondence:
Dr. Ban J. Qasim,
Department of Pathology and Forensic Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq.
E-mail: dr.banqasim@yahoo.com

ABSTRACT

Background/Aim: Diagnosis of Hirschsprung's disease (HD) can be hard and requires good experience, principally for pathologists who infrequently encounter the disease. However, diagnosis is not always possible with hematoxylin and eosin (H and E) because staining has limitations in the identification of immature ganglion cells in neonates and the submucosal area. **Aim:** To assess the diagnostic role of calretinin immunostaining in HD in comparison to neuron-specific enolase. **Patients and Methods:** Formalin-fixed paraffin tissue blocks of full-thickness distal colonic and rectal biopsies for 48 patients who clinically presented with symptoms suspicious for HD were collected for the period from December 2012 to January 2016. All biopsies were already studied by routine H and E histopathological examination for the presence or absence of ganglion cells. Further confirmation of ganglion cells and nerve fibers was performed by immunohistochemical study for neuron-specific enolase and calretinin, respectively, in a private pathology laboratory. **Results:** According to the histopathological assessment, cases with absent ganglionic cells were considered to be HD, which comprised 40 cases out of the total 48 cases. The mean age for HD cases was 19.43 months. The male-to-female ratio in HD cases was 2.34:1. All HD cases showed negative expression of calretinin in small nerve fibers of the lamina propria, musularis mucosae, and submucosa, and negative expression of neuron-specific enolase in ganglionic cells. The sensitivity, specificity, positive predictive value, and negative predictive values for both the markers in the confirmation of diagnosis of HD were all 100%. **Conclusion:** Calretinin immunostaining, similar to that of neuron-specific enolase, is a highly sensitive and specific diagnostic aid to histopathological examination in suspected HD.

Key Words: Calretinin, full-thickness distal colonic and rectal biopsies, Hirschsprung disease, immunostaining, NSE

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Hirschsprung's disease (HD) is a congenital abnormality of the nervous system in the bowel characterized by the absence of ganglion cells from submucosal (Meissner) and myenteric (Auerbach) nerve plexus. Its histopathological diagnosis is based on the absence of these cells from the distal rectum and a variable length of contiguous bowel. HD is an important clinical differential diagnosis in infants and children presenting with severe constipation.[1] One of the approaches is to evaluate multiple hematoxylin and eosin (H and E)-stained levels from each paraffin-embedded biopsy. This technique is applied in most pediatric pathology laboratories. The reliability of this method depends on the observer's ability to accurately distinguish a ganglion cell based on its H and E.[2,3] Although no universal agreement regarding the number of histological sections required for the diagnosis of HD has been approved, previous works relied on the histopathological examination of 50 serial sections stained with H and E.[4,5]

However, in some cases, the diagnosis of aganglionosis may be difficult on routine H and E-stained histologic

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sections only. HD remains a challenging diagnosis, especially among general surgical pathologists who assess these cases occasionally and are insufficiently experienced. In the neonatal period, submucosal ganglionic cells may not be easily identifiable because they are classically small and undifferentiated. Characteristic neuronal nuclear and cytoplasmic features may not be evident.[6]

Acetylcholinesterase (AChE) histochemistry has been a widely used ancillary technique since the 1970s. However, this technique has limitations such as the requirement of frozen section processing, interpretative difficulties, equivocal/false positive results, and technical challenges.[7]

Many institutions started gaining experience with calretinin immunohistochemical stain, as an additional diagnostic tool for HD.[1,8-11] The immunohistochemical detection of neuron-specific enolase (NSE) in mucosal–submucosal rectal biopsies is suitable to exclude HD. Furthermore, high sensitivity of NSE can be aided with another high specific marker for the diagnosis of HD.[12]

The aim of the present work was to assess the diagnostic role of calretinin immunostaining in HD in comparison to NSE.

PATIENTS AND METHODS

Formalin-fixed paraffin tissue blocks of distal colonic and rectal biopsies for 48 patients who clinically presented with symptoms suspicious for HD (constipation, delayed passage of meconium, abdominal distention, etc.) were collected for the period from December 2012 to January 2016. All biopsies were already studied by routine H and E histopathological examination for the presence or absence of ganglion cells. Further confirmation for the detection of ganglion cells and nerve fibers was performed by immunohistochemical study for NSE and calretinin, respectively, in a private pathology laboratory.

Histopathological assessment

All 48 biopsies were full-thickness distal colonic and rectal biopsies. Inadequate biopsies, i.e., biopsies lacking proper thickness of muscularis layer, were rejected from the study. Assessment of (submucosal Meissner and myenteric Auerback) ganglion cells was initially made using multiple levels' serial sections histology technique. Fifty serial sections from properly oriented biopsies were examined by routine H and E stained slides.[4,5]

Immunohistochemical assessment

Cases were stained for (NSE and calretinin) using the VENTANA (ROCHE) BenchMark-XT computerized automated system and the *ultra*View Universal DAB Detection Kit. A total of 4 μm thickness tissue sections were used.

The antibodies used were calretinin rabbit monoclonal antibody (clone SP65) and NSE mouse monoclonal antibody (clone E27).

The *ultra*View Universal DAB Detection Kit detects specific mouse and rabbit primary antibodies, bound to an antigen, in paraffin-embedded tissue sections. The specific antibody is located by a cocktail of enzyme-labeled secondary antibodies (HRP Multimer). The complex is then visualized with hydrogen peroxide substrate and 3,3'-diaminobenzidine tetrahydrochloride (DAB), a chromogen, which produces a brown precipitate that is readily observed by light microscopy. The principal steps of the procedure are illustrated in Figure 1.

The staining protocols followed for both immun-stains (NSE and calretinin) were in accordance with the standard staining protocols of VENTANA (ROCHE) BenchMark-XT system for each antibody. The immunohistochemistry slides were blindly reviewed by two pathologists, independently followed by a common review for agreement.

Neuron-specific enolase

For NSE, the cases were recorded either as positive or negative; a positive ganglion cell categorization was given to an unequivocal strong cytoplasmic NSE stain of the ganglion cells, otherwise the case was scored negative.

Calretinin

For calretinin, the examination was focused on the staining pattern of small nerve fibers in the lamina propria, musularis mucosae, and submucosa (exclusion for mast cells which could be stained for calretinin was made). Likewise, the cases were recorded either positive or negative; a positive nerve fibers categorization was given to an unequivocal strong calretinin stain of small nerve fibers located as above, otherwise the case was scored negative.

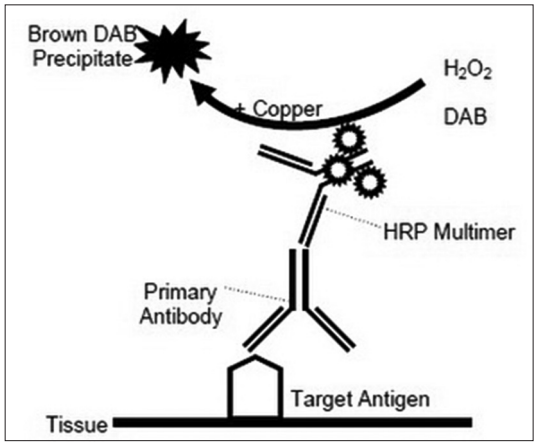

Figure 1: UltraView Universal DAB Detection Kit Reaction

Statistical analysis

The statistical analysis was performed by using GraphPad Prism ® version 6, San Diego, California. Numerical data was described as mean and standard deviation. Categorical data was described as count and percentage. Crosstab model was used to estimate association between studied markers and final diagnosis. Pearson correlation was used to estimate the correlation between studied markers. Further, sensitivity, specificity, positive predictive value, negative predictive value, and kappa index were calculated to estimate the diagnostic effectiveness of studied markers.

RESULTS

In the present study, the mean age for HD cases was 19.43 months. Male-to-female ratio in HD cases was 2.34:1. Histopathological diagnosis of HD depended on the absence of ganglionic cells in submucosal Meissner and myenteric Auerback plexus. To confirm this interpretation, sections from HD and non-HD cases were immunostained with calretinin and NSE. All HD cases showed negative expression of calretinin in small nerve fibers of the lamina propria, musularis mucosae, and submucosa, and negative expression of NSE in ganglionic cells. On the other hand, all non-HD cases (diagnosed histopathologically by the demonstration of ganglionic cells) revealed positive expression of both calretinin and NSE. The sensitivity, specificity, positive predictive value, and negative predictive values for both markers in the confirmation of diagnosis of HD were all 100%. Analysis of the correlation between the two markers by Pearson test demonstrated that all NSE negative cases were negative for calretinin and the same for positive cases in which Pearson's correlation value was 1. The results showed that there was no association between gender type and immunohistochemical expression of calretinin and NSE among all cases.

Descriptive analysis

Patients' age ranged from 1 month to 9 years (mean ± SD = 21.9 ± 23.74 months) and male-to-female ratio was 2,43:1 [Table 1].

According to the histopathological assessment of (submucosal Meissner and myenteric Auerback) ganglion cells in multiple levels H and E-stained serial sections of the distal colon and rectum; cases with absent ganglionic cells were considered HD, which comprised 40 cases out of the total 48 case enrolled in the present study [Figure 2]. The remaining 8 cases with excluded HD showed the presence of ganglionic cells in the H and E-stained serial sections [Figure 3].

The mean age for HD cases was 19.43 months. There was no statistically significant difference in the age groups between HD and HD excluded cases ($P = 0.314$). According to gender, male cases were 28 (70%) in HD whereas 6 cases (75%) were HD excluded males without significant difference ($P = 0.572$). Male-to-female ratio in HD cases was 2.34:1 [Table 1].

Diagnostic value of neuron-specific enolase and calretinin immunohistochemical expression in Hirschberg disease

According to the results of NSE and calretinin immunohistochemical expression in Table 2, it was noticed that all HD cases were both NSE and calretinin negative (40 cases) [Figures 4 and 5] and all HD excluded cases were both NSE and calretinin positive (8 cases) [Figures 6-8]. However, NSE and calretinin expression were 100% sensitive and specific for the discrimination of ganglionic from aganglionic bowel, with kappa index of 1.

Table 1: Descriptive analysis of age and gender in the studied cases

	Final diagnosis		Total	*P*
	HD	HD excluded		
Age (months)				
Mean	19.43	34.25	21.9	0.366^{NS}
SD	17.68	42.82	23.74	
Minimum	1	1	1	
Maximum	72	108	108	
Age groups (months)				
≤24	31 (77.5)	5 (62.5)	36 (75)	0.314^{NS}
>24	9 (22.5)	3 (37.5)	12 (25)	
Gender (%)				
Male	28 (70)	6 (75)	34 (70.8)	0.572^{NS}
Female	12 (30)	2 (25)	14 (29.2)	
Total	40	8	48	

NS: None statistical significant difference (P>0.05). SD: Standard deviation, HD: Hirschsprung's disease

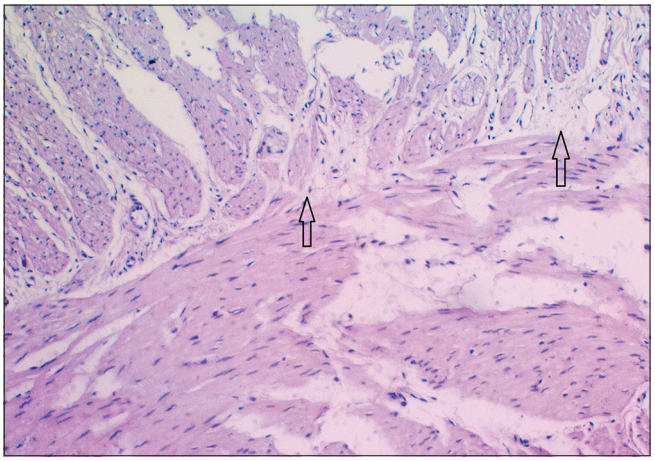

Figure 2: Hirschsprung disease; absence of myenteric plexus (Auerbach's plexus) ganglion cells along the interface between the two layers muscularis layer of colon (arrows). Hematoxylin and eosin stain, ×10

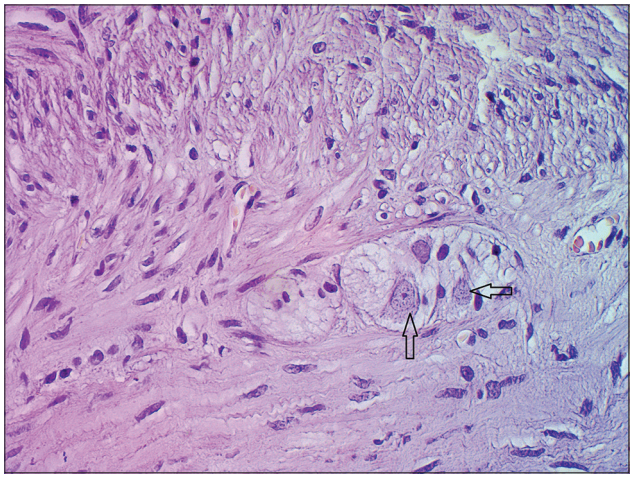

Figure 3: Normally occurring myenteric plexus (Auerbach's plexus) ganglion cells between the two layers muscularis layer of colon; large pyramidal shaped cells with large vesicular nucleus and prominent nucleolus (arrows). Hematoxylin and eosin stain, ×40

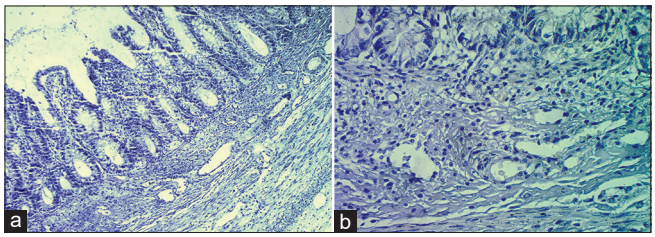

Figure 5: Hirschsprung disease with complete absence of staining of calretinin in small nerve fibers of lamina propria and submucosa layers. (a) power ×10; (b) power ×40

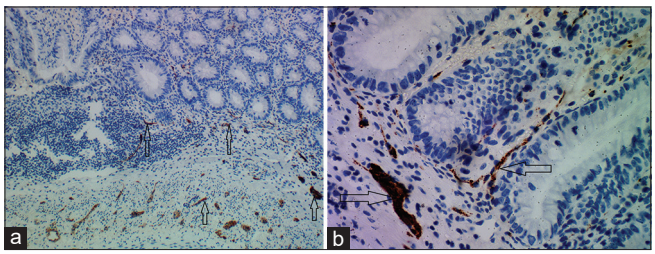

Figure 7: Calretinin: normal presence and distribution in lamina propria and submucosa layers of small nerve fibers showing strong brown cytoplasmic staining pattern for calretinin (arrows). (a) power ×10; (b) power ×40

Further analysis to explore the possible relationship between both markers was done. For this analysis, cross-table model with Pearson correlation were used. The results in Table 3 show that all NSE negative cases were negative for calretinin and the same for positive cases in which Pearson's correlation value was 1.

The results in Table 4 show that there was no association between gender type and immunohistochemical expression of calretinin and NSE among all cases. For both the markers, 28 (82.4%) male cases were negative while only 6 (17.6%) were positive. Concerning female cases, 12 (85.7%) were

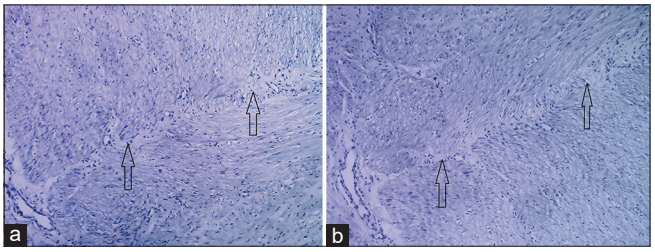

Figure 4: Hirschsprung disease showing negative immunohistochemical staining of neuron specific enolase and calretinin (same case) with absence of myenteric plexus (Auerbach's plexus) ganglion cells along the interface between the two layers muscularis layer of colon (arrows). (a) Neuron specific enolase (×10), (b) calretinin (×10)

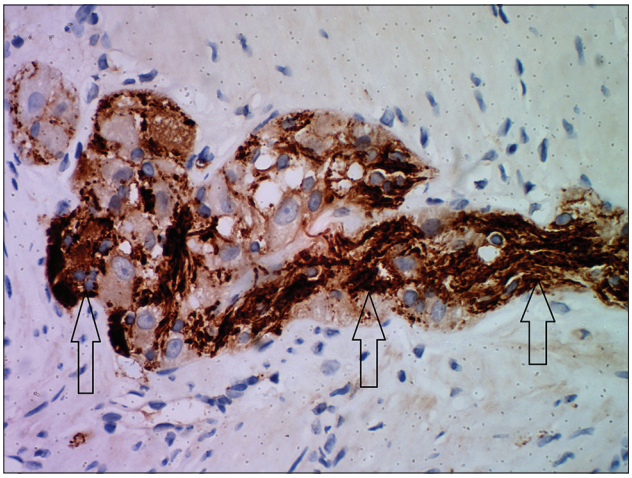

Figure 6: Neuron specific enolase: normal staining pattern for myenteric plexus (Auerbach's plexus) ganglion cells between the two layers muscularis layer of colon; strong brown cytoplasmic stain for a group of ganglion cells (arrows), ×40

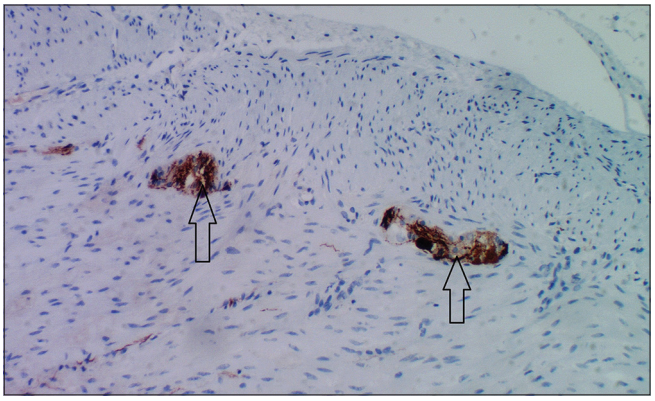

Figure 8: Normal staining pattern of calretinin for myenteric plexus (Auerbach's plexus) ganglion cells between the two layers muscularis layer of colon; strong brown cytoplasmic stain for two groups of ganglion cells (arrows); ×10

negative and only 2 (14.3%) were positive ($P = 0.572$). Furthermore, there was no association between age groups and calretinin or NSE expression in all cases. Among calretinin or NSE negative cases, 31 cases (77.5%) were equal

Table 2: Neuron-specific enolase and calretinin expression according to the final diagnosis of Hirschsprung's disease

	Final diagnosis (%)		*P*	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %	Kappa index
	HD	HD excluded						
NSE								
Negative	40 (100)	0 (0)	<0.001	100 (91.24-100)	100 (67.56-100)	100 (91.24-100)	100 (67.56-100)	1
Positive	0 (0)	8 (100)						
Total	40	8						
Calretinin								
Negative	40 (100)	0 (0)	<0.001	100 (91.24-100)	100 (67.56-100)	100 (91.24-100)	100 (67.56-100)	1
Positive	0 (0)	8 (100)						
Total	40	8						

HD: Hirschsprung's disease, NSE: Neuron-specific enolase

Table 3: Cross-table correlation between neuron-specific enolase and calretinin expression

	NSE		Total
	Negative	Positive	
Calretinin			
Negative			
Count	40	0	40
Percentage	100.0	0.0	83.3
Positive			
Count	0	8	8
Percentage	0.0	100.0	16.7
Total			
Count	40	8	48
Percentage	100.0	100.0	100.0
P	<0.001		
Pearson correlation	1.000		

NSE: Neuron-specific enolase

Table 4: Association between age and gender with calretinin and neuron-specific enolase expression

	Calretinin (%)		NSE (%)		Total
	Negative	Positive	Negative	Positive	
Sex					
Male	28 (82.4)	6 (17.6)	28 (82.4)	6 (17.6)	34
Female	12 (85.7)	2 (14.3)	12 (85.7)	2 (14.3)	14
P	0.572^{NS}		0.572^{NS}		
Age groups (months)					
≤24	31 (77.5)	5 (62.5)	31 (77.5)	5 (62.5)	36
>24	9 (22.5)	3 (37.5)	9 (22.5)	3 (37.5)	12
P	0.314^{NS}		0.314^{NS}		

NS: None statistical significant difference (*P*>0.05). NSE: Neuron-specific enolase

or below 2 years of age and only 5 (62.5%) were positive for the same age group.

DISCUSSION

Lack of ganglion cells in colonic neural plexus is required for the pathological diagnosis of HD. Immunohistochemical staining of calretinin assist in the detection of small immature ganglion cells through intense staining of ganglia, facilitating the recognition of small immature ganglion cells. Nevertheless, the assessment of many cases is still difficult, thus requiring demanding repeated deeper sections.[13]

Zuikova *et al.* stated that calretinin immunohistochemical technique is less challenging and can be interpreted more easily than AChE. It shares with AChE the lesser requirement to look for ganglion cells in several serial sections of tiny rectal tissue biopsy. They also mentioned that the application of combined one positive (AChE) and one negative (calretinin) could take full advantage of the precision to diagnose HD.[14]

In the present study, all HD cases showed negative expression of calretinin in small nerve fibers of the lamina propria, musularis mucosae, and submucosa, and negative expression of NSE in ganglionic cells. On the other hand, all non-HD cases (diagnosed histopathologically by the demonstration of ganglionic cells) revealed positive expression of both calretinin and NSE.

In agreement to these results, Barshack *et al.* summarized that aganglionic segments revealed absence of calretinin expression in ganglion cells and in the nerve fiber in HD, and conversely calretinin expression was positive in both ganglion cells and nerve fibers in ganglionic areas of HD and normal colon.[13]

Likewise, Małdyk *et al.* reported a study in 2014 including results that are concordant with the present one, showing that expression of calretinin was positive in all rectal biopsies with ganglionic cells while negative expression was noticed in all aganglionic segments, thus concluding that immunohistochemical staining of calretinin is a valuable adjunct to histopathology in the diagnosis of HD.[6]

Lim *et al.* recorded two false negative results out of the 27 patients with HD, caused by technical overstaining

and punctate immunoreactivity of deep submucosal hypertrophied nerves. They concluded that immunostaining with calretinin is a reliable ancillary technique in the investigation of HD.[7]

Another study performed by Guinard-Samuel *et al*. demonstrated that difficulties faced using combined histopathological examination and staining with acetylcholinesterase can be bypassed through immunostaining with calretinin, and that all HD can be diagnosed accurately without false positive results.[15]

In an institutional experience, Alexandrescu *et al*. affirmed in their work published in 2013 that calretinin immunohistochemical test is a dependable diagnostic method for the pathologist when used in combination with histopathological examination, particularly in cases with sparse or immature ganglion cells in colonic submucosa.[8]

Hiradfar *et al*. investigated the expression of calretinin in colonic sections of HD in comparison to control cases and found that, in both HD patients and control cases, calretinin immunostaining was positive in the nerve fibers of the lamina propria, submucosa, and muscularis propria. Ganglion cells in submucosa and muscularis propria revealed positive calretinin expression in all specimens of both control group and ganglionic segments of HD cases. Immunohistochemical expression of calretinin was negative in all but 2 cases in the muscularis propria nerve fibers of the aganglionic segments. Sensitivity and specificity of this method for the diagnosis of HD in full thickness specimens of intestinal wall were 93.3% and 100%, respectively, with a positive predictive value of 100% and negative predictive value of 93.8%.[10] In the same manner, Mukhopadhyay *et al*. reported that sensitivity of calretinin immunohistochemistry for ganglion cells detection was 100% and that the specificity was 97.44%, with positive and negative predictive value of 84.62% and 100%, respectively. Another study showed that the sensitivity of calretinin reactivity in ganglion cells was 90.5% and specificity was 92.9%.[3]

Results of Kaçar *et al*., which are concordant with the present work, revealed a great equivalence between histopathological assessment and calretinin immunostaining, concluding that immunohistochemical testing of calretinin has high sensitivity and specificity for the diagnosis of HD, reducing the requirement for repetitive biopsies and unnecessary sectioning for suction biopsy, full thickness biopsy, as well as resection specimen.[1] Similarly, Gonzalo *et al*. found in their retrospective study that all patients without HD had positive immunohistochemical expression of nerve fibers in lamina propria or muscularis mucosae, and all Hirschsprung patients showed negative calretinin expression concluding that immunohistochemical testing of calretinin is quite supportive in triaging additional workup based on clinical suspicion.[16]

Regarding usefulness role of NSE in the diagnosis of HD, Nogueira *et al*. compared the diagnostic role NSE immunohistochemical expression in HD with H and E staining in consecutive sections, and accomplished that both H and E and NSE immunostaining had identical value in the assessment of neurons in sections of rectal wall of patients with clinically suspected HD.[17] MacKenzie *et al*. stated that staining for NSE is helpful to detect immature ganglion cells in paediatric large intestine.[18] In another published article, the authors showed that NSE immunostaining produced intense staining of ganglion cells' perikarya, significantly assisting in identification of small immature forms. They concluded that NSE immunohistochemical testing may help in the elucidation of rectal mucosal biopsies when HD is suspected.[19]

In 2006, Torabizadeh *et al*. reported that sensitivity, specificity, efficiency, and positive and negative predictive values in the diagnosis of HD in NSE method were 100%, 84.2%, 89.1%, 81.8%, and 100%, respectively, and concluded that, with positive immunohistochemical expression of NSE, detecting ganglion cell absolutely excludes HD, however, absence of ganglion cell confirms 81.8% of HD cases.[12]

Robey *et al*. retrospectively reviewed biopsy specimens from patients with suspected and proven HD, and concluded that NSE immunostaining is of value in identifying ganglion cells in suspected cases of HD.[20]

CONCLUSION

The present study concludes that calretinin immunostaining, like that of NSE, is a highly sensitive and specific diagnostic aid to histopathological examination in suspected HD.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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