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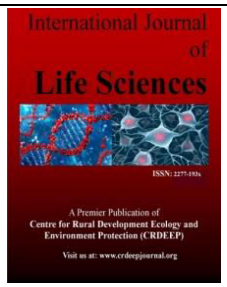

Full Length Research Paper

The Effect of Calretinin Immunostaining as Adjunctive to Hematoxylin and Eosin in Improving the Diagnosis of Hirschsprung Disease

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ABSTRACT

Introduction: Hirschsprung's disease (HD) is a congenital intestinal motility disorder with absence of ganglion cells in the colonic wall. It is a congenital multigenetic disorder which pathogenesis is not yet fully understood. Objective: This study was undertaken with an objective to *determine the diagnostic value of calretinin IHC in detecting aganglionosis (HD) in rectal biopsy both Full Thickness and split thickness and its relation to Hematoxylin and Eosin and the interobserver difference in detection of aganglionosis as this difference render the diagnosis of HD is not an easy process. Patient and methods: The study was conducted in the period between January 2017 to January 2019 on patients from Ain Shams University and Ahmed Maher Teaching hospital.33 patients suspect of having HD were biopsied as full and split thickness rectal biopsy for each patient both the biopsies stained by Hematoxylin and Eosin and calretinin all in the pediatric age group and complaining of chronic constipation that started since birth in most of the cases. Results: The results were collected in a prospective study, The IHC slides and H&E were evaluated by two pathologists. The sensitivity of calretinin IHC stain were calculated in correspondence to the clinical data and the H&E staining in both Full and split thickness biopsies. The agreement of pathologists to the Calretinin IHC and H&E staining were calculated. It was noted that Calretinin IHC had high sensitivity in full thickness (95.8%) rectal biopsy followed by calretinin IHC in split thickness rectal biopsy (91.7%) in comparison to H and E which had 87.5 percent in full thickness and lastly the H&E split thickness (58.3%) and the interobserver agreement was highly significant in Calretinin IHC than H&E as measured by the (kappa agreement). Conclusion: Calretinin IHC when combined with H&E showed to increase the diagnostic accuracy of aganglionosis and limit the interobserver difference to overcome the misdiagnosis.*

Introduction

Hirschsprung's disease occurs in approximately 1 per 5,000 live births. This disease is characterized by absence of parasympathetic ganglion cells in the intermuscular myenteric (Auerbach) and submucosal (Meissner) plexuses of the affected colon, causing a sustained contraction of that segment. In approximately 80% of cases, the aganglionic segment involves the rectum and the sigmoid colon only, whereas in 20% of cases, the aganglionic segment involves the more proximal bowel, sometimes extending to variable lengths of the small intestines [1]. The diagnosis of Hirschsprung's disease is based on a combination of clinical features, radiological appearance of the bowel and histopathological features on rectal biopsies that include absence of ganglion cells and abnormally hypertrophic nerves [2].

The assessment of serial hematoxylin-eosin (H&E) stained rectal biopsies has many difficulties, including superficial samples with limited submucosa or samples from too distal sites of the anorectum in addition to the physiological paucity of ganglion cells, which all are considered to be challenging problems in identification of ganglion cells, especially in

neonates, owing to immaturity of the enteric nervous system [3]. For these reasons, over the years conventional histology has been supplemented with histochemical and immunohistochemical methods to assist the identification of ganglion cells toguarantee reaching an accurate diagnosis [4].

Regarding immunohistochemistry, several immunohistochemical markers were studied to aid the diagnosis of HD, including S-100 protein, Glial Fibrillary Acid Protein (GFAP), Glucose Transporter 1 (GLUT-1), Microtubule Associated Protein 5 (MAP-5) and others. But, none of them has been widely adopted [5].

Calretinin is a vitamin D dependent calcium binding protein. The anticalretinin monoclonal antibody stains most of ganglion cells as well as small intrinsic nerve fibers of the submucosal and myenteric plexuses in the normal colon. Loss of calretinin-immunoreactive ganglion cells and nerves has been suggested to be of value in the diagnosis of Hirschsprung's disease [6].

Patient and method

The study was comparative diagnostic intervention study. The study was conducted on patients from Ahmed Maher Teaching hospital and Pediatric Surgery Department, Ain Shams University Hospitals from January 2017 to January 2019.

Sample size: 27 cases for detection of sensitivity of each test.

Methodology

That was conduct in the period from January 2017 to January 2019. All Patient suspect of diagnosis of Hirschsprung disease in any pediatric age group were included in the study during this period but Redo or previously biopsied patient were excluded.33 cases were included all complaining of chronic constipation that started since birth. All patients were preoperatively examined and prepared for operative biopsy. The preoperative investigations were within normal values. Informed consent was taken from the caregivers of the cases of the study.

All the patients' data including age, sex and clinical presentation were collected by the researcher from patient medical files. Biopsies were taken from all cases under general anesthesia with uneventful postoperative period. All Cases were submitted to Full thickness and split thickness rectal biopsy by open and punch techniques respectively. The conventional H&E stain was used as the initial step in evaluation of all the studied cases.

Rectal biopsy specimens divided into split thickness specimens which consisted of mucosa (surface epithelium, lamina propria and muscularis mucosa) and submucosa with no muscularis propria or serosa and full thickness specimens which consisted of consisted of mucosa (surface epithelium, lamina propria and muscularis mucosa) and submucosa with muscularis propria. These biopsies were evaluated for presence or absence of ganglion cells in the submucosal plexus (Meissner's plexus) in split thickness and the submucosal plexus (Meissner's plexus) and in the muscularis propria plane (Auerbach's plexus).

If present, ganglion cells were found separately or in clusters and they were located anywhere in the submucosa either directly under the muscularis mucosa or deeper into the submucosa or in the muscularis propria (figures3&4).

Identification of ganglion cells was done through recognition of their unique morphology as being oval large cells with eccentric vesicular basophilic nuclei, single prominent often eosinophilic nucleoli and abundant eosinophilic cytoplasm (figure 3).

Both rectal biopsies were also examined for the presence of hypertrophied thickened nerve fibers in the submucosa and in the muscularis propria plane that were identified as oval or round structures with wavy basophilic nuclei and fibrillary light eosinophilic cytoplasm (figures 1&2).

Both the full thickness and the split thickness examined and reported for the clinical decision for the definitive management of the patient and the results recorded for the study.

Detection of ganglion cells (especially immature forms or ganglion cells that existed singly and not in clusters) using H&E was sometimes a very strenuous process, so when the presence or absence of ganglion cells could not be confirmed by the H&E stain, these cases were labeled as *"Suspicious"*.

Calretinin

To evaluate Calretinin staining, mesothelium of the pleural tissue was selected as positive control and the antibody was omitted from the staining process for negative control. Positivity for Calretinin was detected as brown staining on the counter background Hematoxylin blue stain (figure 7,8). Calretinin immunoreactivity was observed in the ganglion cells of the submucosal and intermuscular plexuses, the pattern of expression was dense nuclear and cytoplasmic (figure 7,8).

Calretinin expression was also observed in the fine nerve fibrils of the lamina propria, muscularis mucosa and submucosa, the staining pattern was linear, cytoplasmic and granular. Calretinin positivity was sometimes observed in the cytoplasm of large submucosal nerves and in some submucosal structures (mainly inflammatory cells) the muscularis propria plane. All cases were evaluated for positivity of Calretinin, and the absence or presence of ganglion cells was noted for each case. Both the full thickness and split thickness examined and reported for the study.

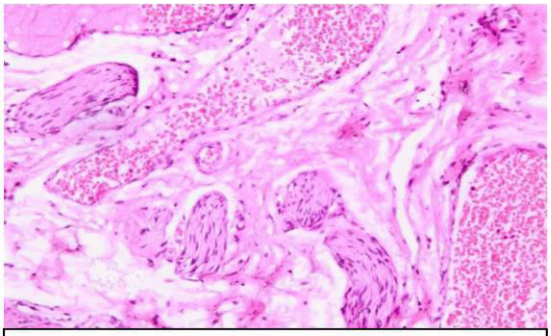


Figure (1) shows H&E negative case without ganglia and nerve bundle hypertrophy HD case in submucosa (H&Ex200).

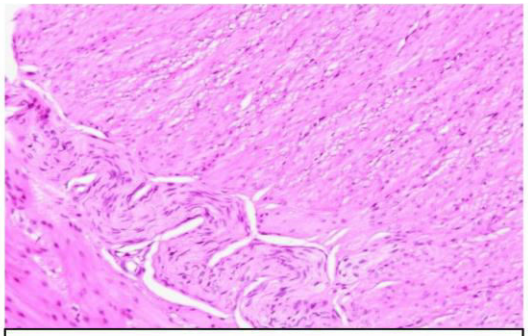


Figure (2) shows H&E negative case without ganglia and nerve bundle hypertrophy HD case in muscle (H&Ex200).

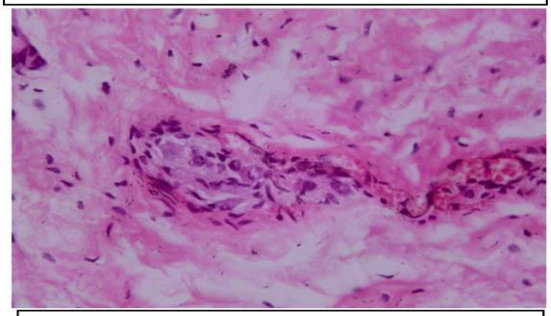


Figure (3) shows H&E positive case with ganglia presentnon-HD case in submucosa (H&Ex400).

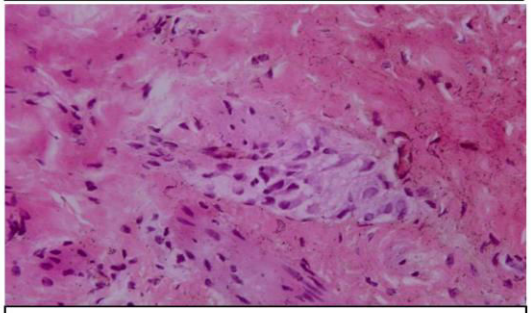


Figure (4) shows H&E positive case with ganglia present non-HD case in muscle plane(H&Ex400).

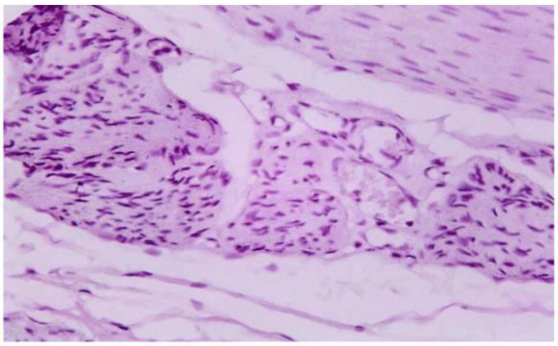


Figure (5) shows calretinin negative expression HD case in muscle (IHCx400).

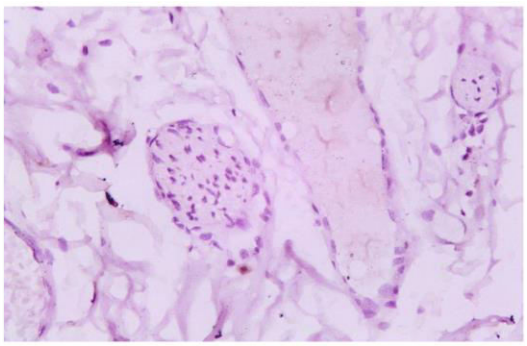


Figure (6) shows calretinin negative expression HD case in submucosa (IHCx400).

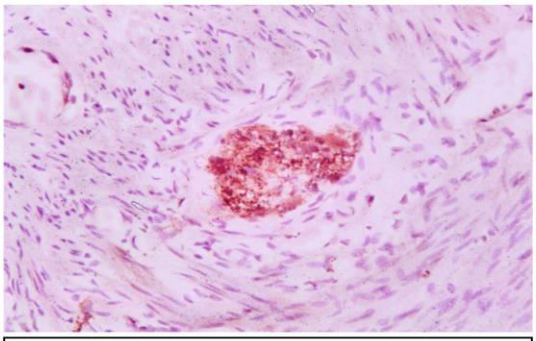


Figure (7) shows Calretinin positive expression case with ganglia non-HD case in muscle plane (IHCx400).

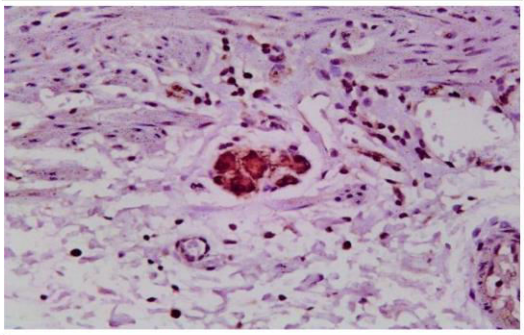


Figure (8) shows Calretinin positive expression case with ganglia non-HD case in submucosa (IHCx400).

Results and Discussion

Holland et al. in 2011, stated that accurate diagnosis depends on many factors including; the site of biopsy, representativeness of the samples taken, the number of specimens, and finally the pathologist's skill. They added that the pathologist needs a good biopsy that contains adequate amounts of submucosa and is taken at least 2 cm away from the dentate line (to avoid sampling from the physiological aganglionic zone in the first part of the anal canal). They believe that with optimal conditions, diagnostic sensitivity can even reach 100%.

The basic well known obligate histopathological diagnostic feature of the disease is the total lack of ganglion cells in the submucosal (Meissner) and intermuscular (Auerbach) nerve plexuses of the intestinal wall. The presence of multiple hypertrophic nerve fibers is observed in many (but not all cases) and helps establish the diagnosis [7,8].

White and Langer, in 2000, stated that the presence or absence of submucosal ganglion cells correlates with the status of ganglion cells in the adjacent myenteric plexus, examination of suction biopsies allows only the assessment of the submucosal plexus which can make the diagnosis more difficult [9].

Maldyk et al., in 2014, reported that the conventional H&E stain which is the first histopathological tool for diagnosis has many limitations including paucity of submucosa (especially in suction biopsies), crush artifact and in the neonatal period (when most of the cases are presented and diagnosed); submucosal ganglion cells may not be easily recognizable because their maturation is incomplete at birth and they tend to take unipolar or bipolar forms that are typically small, undifferentiated with small or absent nucleoli and scarce cytoplasm which is different from the classical phenotype of the mature ganglion cells so they can be very easily confused with endothelial cells, histiocytes or lymphoid cells [10].

Karim et al., in 2006 and Kapur et al., in 2009, also thought that the diagnosis is a burdensome mission adding several more facts that increase complexity of the diagnostic process for example; submucosal ganglia are normally relatively widely separated from one another and they are most abundant along the internal layer of the muscularis propria, in the deep portion of the submucosa that is not always sampled well by the rectal biopsy technique such that time consuming preparation and evaluation of numerous H&E sections (sometimes up to examination of the entire block) is required in some cases to identify unequivocal ganglion cells. Finally, the hypertrophic extrinsic nerves are not always present, so they are an incompletely sensitive marker of the malformation [8,11].

According to De la Torre and Santos, in 2012, errors in the histological diagnosis arise from the biopsy itself (surgeon dependent) or its processing and examination (pathologist dependent). Wrong diagnoses in rectal biopsies are common and lead to either over or under diagnosis. The first situation, results in unnecessary colonic resection and pull-through and the second causes a delayed diagnosis. They believe that the problem of the histopathological diagnosis is that the pathologist should demonstrate something that " does not exist" which means that they have to confirm the absence of ganglion cells in the submucosal and myenteric plexuses and the dilemma is resolved for any pathologist when ganglion cells are definitely identified regardless which histological technique was used [12].

In the current study, H&E staining was performed for all 33 cases; full and split thickness rectal biopsies and were examined and both blocks stained H&E and Calretinin.

Regarding rectal biopsies Full thickness rectal biopsies the H&E results was as follow as in table (1):

In 22 cases (66.6%) no ganglion cells could be detected in the submucosa or the intermuscular plexus after examination of many serials and these cases were interpreted as negative for ganglion cells. In addition to the absence of ganglion cells, multiple thick nerve trunks were also observed (Figures 1&2).

In 5 cases (15%) ganglion cells were identified in the submucosa or in the intermuscular plexus and therefore these cases were interpreted as positive for ganglion cells and the diagnosis of HD was excluded (Figures 3&4).

In 6 cases (18%) the results were suspicious meaning that based on morphology, no definite ganglion cells could be detected, and the presence or absence of ganglion cells could not be confirmed by H&E. Thus, the final diagnosis in these cases could not be reached using H&E alone.

Table (1): - Results of H&E full thickness in diagnosis and exclusion of hirschsprung disease.

H&E Full thickness	**HD**	**Non-HD**	Total	
	Diseased	Not diseased		
Negative	21	1	22	Pearson Chi-square **20.629[a]**
% within diseased	**87.5%**	11.1%		
Positive	0	5	5	
% within diseased	0%	**55.6%**		
Suspicious	3	3	6	Asymp. sig P 2 sided **.000**
% within diseased	12.5%	33.3%		

Regarding rectal biopsies Split thickness rectal biopsies the H&E results was as follow as in table (2):

- In 15 cases (45.5%) no ganglion cells could be detected in the submucosa after examination of many serials and these cases were interpreted as negative for ganglion cells. In addition to the absence of ganglion cells, multiple thick nerve trunks were also observed (Figures 1&2).
- Ganglion cells could not identified in the submucosa and therefore no case interpreted as positive for ganglion cells or the diagnosis of HD was excluded.

- In 18 cases (54.5%) the results were suspicious meaning that based on morphology, no definite ganglion cells could be detected, and the presence or absence of ganglion cells could not be confirmed by H&E. Thus, the final diagnosis in these cases could not be reached using H&E in split thickness biopsy.

Table (2): - Results of H&E split thickness in diagnosis and exclusion of hirschsprung disease.

H&E split thickness	**HD** Diseased	**Non-HD** Not diseased	Total	
Negative	14	1	15	Pearson Chi-square **5.887**[b]
% within diseased	**58.3%**	11.1%		
Positive	0	0	0	
% within diseased	0%	0%		
Suspicious	10	8	18	Asymp. sig P 2 sided **.015**
% within diseased	12.5%	33.3%		

Summarizing the previous results; H&E didn't provide a definite answer in 6 cases ((18%) of the total number) of Full thickness rectal biopsies, and in 18 Split thickness rectal biopsies (54.5 %) of the total number in these cases interpretation of H&E sections was considered suspicious and inconclusive.

Alexandrescu et al., in 2013, and several other reports as well confirmed restrictions in H&E diagnosis (these restrictions matched the findings and hardships encountered in our current study relying on H&E alone in the diagnosis or exclusion of HD), so they thought that using ancillary methods such as histochemistry or immunohistochemistry is a must specially in problematic cases [13].

Some institutions use acetylcholinesterase histochemical stain to help guide the pathologists in their diagnosis. Acetylcholinesterase shows abnormally coarse and dense nerve fibers in the muscularis mucosa and submucosa of aganglionic bowel. This is the only auxiliary diagnostic method that is positive in HD [14].

According to Kannaiyan et al., in 2013, the drawbacks of acetylcholinesterase stain is that it necessitates frozen tissue which is not available in all medical centers, it is a difficult histochemical stain that is performed only for suction rectal biopsies, it needs quantitative and qualitative assessment therefore a high degree of subjectivity exists in its interpretation, in addition to the possibility occurrence of false negative and positive results [15].Therefore many authors vote against using histochemistry, a situation that has led to the quest for other methods to aid in diagnosis such as immunohistochemistry which is the other major ancillary approach utilized to complement H&E based histopathology and many different antigens have been investigated in this context such as S100, CD117, CD56, Cathepsin D, BCL2, Synaptophysin, Chromogranin, Microtubule Associated Protein-5, Glucose Transported-1 (GLUT-1), Glial Fibrillar Acidic Protein (GFAP) and Peripherin antibodies, but none of them has gained worldwide recognition in the diagnosis of HD [16,17].

Calretinin is a vitamin D dependent calcium binding protein that functions as a calcium sensor/modulator; it belongs to the EF-hand family which comprises over 150 members. It is expressed in the specific neurons of the central and peripheral nervous systems and its expression is observed in nonneuronal cells as well [18].

Barshacket al., in 2004. found that in the gut it's expressed by a subset of submucosal and myenteric ganglion cells, which project nerve terminals into the mucosa. Therefore, it was proposed by some studies that Calretinin could be of use in diagnosing Hirschsprung's disease [19].

In order to investigate the diagnostic utility of Calretinin in Hirschsprung's disease we conducted a comparative study between Calretinin and H&E where Calretinin expression was studied in all the included cases both in Full thickness and split thickness rectal biopsies.

Regarding rectal biopsies Full thickness rectal biopsies, the Calretinin results was as follow as shown in table (3):

In 23 cases (69.7%) no Calretinin expression was identified in the submucosa or the lamina propria or in the intermuscular plexus indicating the absence of ganglion cells. These cases were interpreted as negative for Calretinin stain (Figure 5&6).

In 9 cases (27.3%) there was strong dense nuclear and cytoplasmic brown staining of the submucosal ganglion cells as well as linear granular cytoplasmic staining of the fine nerve fibrils in the lamina propria, muscularis mucosa and submucosa and in the intermuscular plexus. These cases were interpreted as positive by Calretinin (Figures 7&8).

In one of these non HD cases (3%); A thin strip of muscularis propria was included (a finding that was not encountered in any other rectal biopsy) and a suspected ganglion cells was found in between the muscle wisps although examination of the same section with H&E didn't reveal any ganglion cells.

In a minority of cases, weak and irregular Calretinin staining unsimilar to ganglion cell morphology was found in a few scattered cells in the submucosa, these cells were probably inflammatory cells; mast cells and histiocytes.

Table (3): - Results of Calretinin full thickness in diagnosis and exclusion of hirschsprung disease.

Calretinin Full thickness	**HD** Diseased	**Non-HD** Not diseased	Total	Pearson Chi-square **33.000[a]**
Negative	23	0	23	
% within diseased	**95.8%**	0%		
Positive	0	9	9	
% within diseased	0%	**100%**		Asymp. sig P 2 sided **.000**
Suspicious	1	0	1	
% within diseased	4.2%	0%		

Regarding rectal biopsies Split thickness rectal biopsies the Calretinin results was as follow as shown in table (4):

In 23 cases (69.7%) no Calretinin expression was identified in the submucosa or the lamina propria indicating the absence of ganglion cells. These cases were interpreted as negative for Calretinin stain (Figure 6).

In 5 cases (15.2%) there was strong dense nuclear and cytoplasmic brown staining of the submucosal ganglion cells as well as linear granular cytoplasmic staining of the fine nerve fibrils in the lamina propria, muscularis mucosa and submucosa. These cases were interpreted as positive by Calretinin (Figure 8).

In 5 cases (15.2%) the results were suspicious meaning that based on morphology, no definite ganglion cells could be detected, and the presence or absence of ganglion cells could not be confirmed by calretinin in the submucosa. Thus, the final diagnosis in these cases couldnot be reached using calretinin in split thickness biopsy

Table (4): - Results of Calretinin split thickness in diagnosis and exclusion of hirschsprung disease.

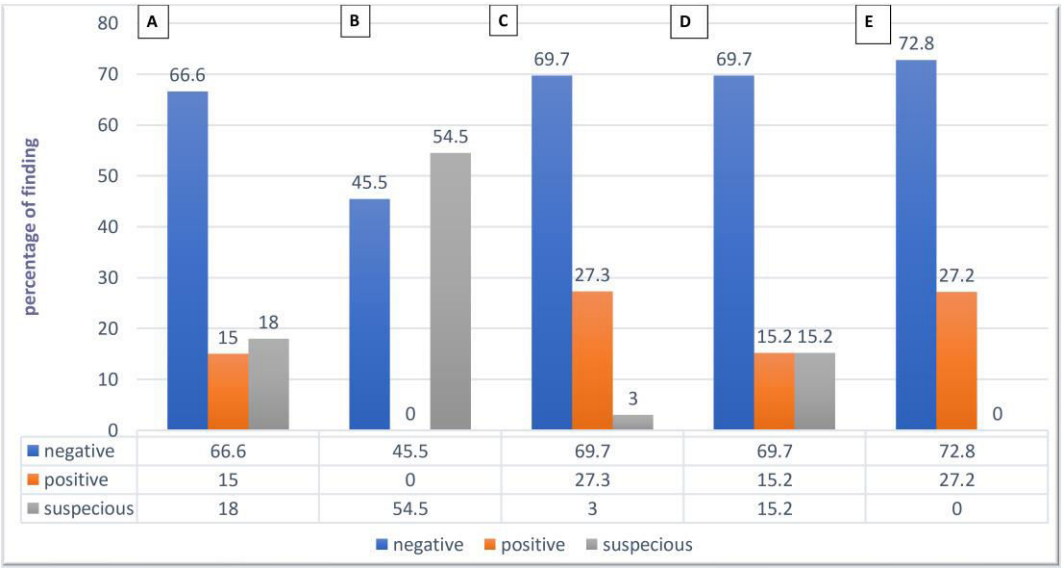
Calretinin Split thickness	**HD** Diseased	**Non-HD** Not diseased	Total	Pearson Chi-square **22.0128[a]**
Negative	22	1	23	
% within diseased	**95.8%**	11.1%		
Positive	0	5	5	
% within diseased	0%	**55.6%**		Asymp. sig P 2 sided **.000**
Suspicious	2	3	5	
% within diseased	8.4%	33.3%		

Our findings regarding Calretinin expression in rectal biopsies were consistent with Guinard-Samuel et al. 2009 who conducted a large series retrospective study including 131 rectal biopsies from children suspected to have HD; they compared Calretinin staining to their conventional methods using H&E and acetylcholinesterase. After examination of Calretinin stained sections, 78 cases were diagnosed as non-HD and 53 cases were diagnosed with HD [20].

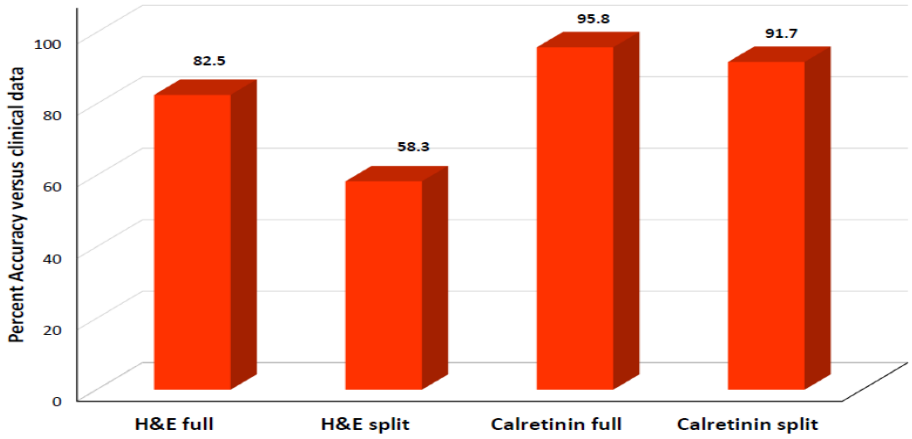
Calretinin based diagnoses in all these cases were later on proved to be correct either by examination of the resected bowel loops from the HD cases or by long term follow in non-HD cases. In their study, Calretinin interpretation matched results obtained by their usual methods in 119 cases and the remaining 12 cases that were initially considered suspicious using the standard technique were accurately diagnosed by Calretinin. They have concluded that Calretinin is efficient in proving the absence of ganglion cells and is superior to AChE and can replace it in diagnosing HD.

Our findings also matched results obtained by De Arruda Lourenção et al. in 2013, who performed a study on Calretinin as well [3]. The study began with a total of 83 patients; they were first investigated with anorectal manometry and/or barium enema as screening tests but only 43 patients were submitted for rectal biopsies. They found absence of Calretinin expression in 14 of 43 cases diagnosing these cases as HD in contrast to H&E that did not reveal ganglion cells in 24 of 43 cases. So, 10 cases were falsely diagnosed as having Hirschsprung's disease by H&E. Therefore, based on the wide disagreement in their results between Calretinin and H&E they concluded that Calretinin showed significantly higher specificity and accuracy values than H&E. Their study also highlighted the accuracy of rectal biopsies over the functional investigations (manometry and enema); that only 14 cases (one third) eventually turned out to have HD although findings obtained with these investigations were compatible with HD in 43 cases.

In the difficult cases where H&E interpretation results could not provide a definite diagnostic decision and report suspicious, Calretinin determined undoubtedly the presence or absence of ganglion cells therefore establishing a solid diagnosis. So, the sensitivity and specificity of Calretinin in detecting ganglion cells in different rectal biopsy specimens of this study high reaching 100% in full thickness rectal biopsy. Moreover, Calretinin had a perfect interobserver agreement and was statistically significant $p < 0.0001$.



Graph (1): demonstrate the Finding of the four type of results.
A: Full thickness rectal biopsy H&E. B: split thickness rectal biopsy H&E .
C: Full thickness rectal biopsy Calretinin. **D**: Split thickness rectal biopsy calretinin.
D: The percentage of diseases or not in correspondence to clinical data.



Graph (2): shows the percent of accuracy of each method of study versus clinical data.

The present study valued numerous advantages in using Calretinin. First, it can be applied to sections from the same formalin fixed paraffin embedded tissue used for conventional diagnosis and not requiring a frozen section. Second; Calretinin showed a great benefit in the analysis of inadequate specimens that contain insufficient amounts of submucosa. Although the diagnosis of HD is still centered on evaluating the absence of ganglion cells, in superficial biopsies this may not be possible therefore in these insufficient biopsies, normal Calretinin reactivity in nerve fibrils in the lamina propria and muscularis mucosa can help in making a pathological assessment, thus ruling out the diagnosis of HD.

This conclusion is consistent with a study that was carried out by Gonzalo and Plesec, in 2103, who were interested in studying Calretinin expression in inadequate biopsies. According to them, up to 17% of all rectal suction biopsies performed for evaluation of HD are considered inadequate [21]. Their work included 17 biopsies that were considered superficial; Calretinin expression was present in the nerve fibrils of the lamina propria, muscularis mucosa and included portions of submucosa in 12 cases, excluding the diagnosis of HD. All these cases were actually confirmed to be non-HD afterwards either by obtaining and evaluation of another more representative rectal biopsy or by follow up for an average of 7 years.

The last advantage of Calretinin is that the marker is either positive or negative for immunoreactivity thus reducing the ambiguity in diagnosis; equivocal or misleading results are rare so there is little interobserver disagreement and no false-positive interpretations.

The final observation obtained from our work is the punctate faint expression of Calretinin in the axons of large extrinsic nerves in the submucosa. This was seen in 5 rectal biopsy cases (16%) and in 8 colectomy specimens in only the distal ends (26.6%). This finding was considered to be of no diagnostic significance as it was observed in ganglionic and aganglionic bowels.

Guinard-Samuel et al., in 2009, in their extensive study also reported this finding but in only one case out of 131 cases examined [20]. And in Kannaiyan et al., 2013 study they found slight Calretinin positivity in 2 cases (out of 60) in some large bundles with no further staining in other areas, and their explanation for this finding was that these sections was taken from the transition zone [15].

Finally, Holland et al., in 2011, and few other reports have emphasized the possibility of false-negative results because of confounding factors such as Calretinin immunoreactivity in mast cells, histiocytes, other inflammatory cells and in the previously mentioned deeper hypertrophic extrinsic nerves, typically found in Hirschsprung's disease [5].

Similar findings were observed in some of the cases in this study, but with thorough examination, the structures uptaking Calretinin stain were excluded from being ganglion cells based on their morphology and the distinction between these cells and ganglion cells was quite easy.

Finally, after reaching the final diagnosis in all cases based on our H&E and Calretinin interpretation results we found that 18 males and 6 females in the study had Hirschsprung's disease, so the male to female ratio in our study was 3:1 which is fairly close to the global HD male to female ratio that is 4:1 [22]. It was noted that Calretinin IHC had high sensitivity in full thickness (95.8%) rectal biopsy followed by calretinin IHC in split thickness rectal biopsy(91.7%) in comparison to H and E which had 87.5 percent in full thickness and lastly the H&E split thickness (58.3%) as shown in graph (2) and the interobserver agreement was highly significant in Calretinin IHC than H&E as measured by the (kappa agreement) P value < 0.005.

Conclusion

Using Calretinin stain facilitated the diagnostic process to a great extent and helped establish a diagnosis in 24 situations (6 full thickness rectal biopsies and 18 split thickness rectal biopsies) where the H&E interpretation was suspicious. The expression pattern is simple, distinct and easy to interpret so it is an excellent option that can be used by pathologists in case of diagnostic doubt of HD. It also reduced the time, effort and number of sections needing to be examined to reach a precise diagnosis and limit the interobserver disagreement and confusion that found in H&E.

Disclosure

Conflict of interest: none

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