RESEARCH ARTICLE

Diagnostic accuracy of haematoxylin-eosin staining in comparison to calretinin and S100 for the assessment of ganglion cells in rectal biopsy

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Abstract

Objectives: To assess the diagnostic accuracy of haematoxylin-eosin staining in clinically suspected Hirschsprung disease, and to compare the findings with calretinin and S100 immunohistochemistry.

Method: The retrospective study was conducted at the AL-Khansaa Teaching Hospital, Nineveh, Iraq, and comprised data from January 2017 to October 2020 of rectal suction biopsies of patients with clinically and radiologically suspected Hirschsprung disease. Histopathology and immunohistochemistry were performed. Data was analysed using SPSS 16.

Results: Of the 114 patients, 74(64.9%) were males and 40(35.1%) were females. Based on histology, 28(24.6%) cases were negative for ganglion cells, and, of them 25(89.2%) revealed nerve bundle hypertrophy. The diagnostic accuracy for the detection of ganglion cell and nerve hypertrophy using haematoxylin-eosin stain was 99.1% and 94.4%, respectively. Correlation of haematoxylin-eosin staining with calretinin and S100 was statistically near perfection ($\kappa = 0.976$ and $\kappa = 0.923$), respectively.

Conclusion: The mainstay to confirm or exclude Hirschsprung disease remains an accurate histopathological evaluation of the haematoxylin-eosin-stained sections of an adequate colorectal biopsy.

Keywords: Hirschsprung disease, Calretinin, S100, Nerve hypertrophy, Rectal biopsy. (JPMA 72: 1123; 2022)

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Introduction

Hirschsprung disease (HD) is a congenital disorder with complex patterns of inheritance.[1,2] The estimated prevalence of the disease is at the level of 1/5000 newborns with 4:1 male-to-female predominance.[2]

If HD is clinically suspected, an adequate rectal biopsy is recommended for establishing the diagnosis, followed by surgical resection of the affected segment pulled through the healthy intestine.[1,2]

Haematoxylin-eosin (HE) remains the gold standard, and is a universally utilised approach for the evaluation of clinically suspected intestinal aganglionosis in rectal biopsies.[3] However, HE has some challenges in that a precise diagnosis depends on the pathologist's experience of analysing multiple serial sections for the presence or absence of ganglion cells.[4,5] Besides, in neonates and infants, there are some difficulties in identifying submucosal ganglion cells with confidence because they are classically immature, small with an irregular distribution.[6] Finally, the surgeon's skill to obtain an adequate rectal biopsy sample the mucosa and submucosa is critical.[7] Therefore, nowadays, numerous institutions use ancillary methods like immunohistochemical (IHC) markers in the identification of ganglion cells and hypertrophied nerve bundles.[7,8]

One of these immune markers is called calretinin, a recent and novel immune-stain utilised as a diagnostic tool for HD. It is a calcium-binding protein which stains ganglion cells of the healthy intestine.[9-11] The nerve fibres also can be highlighted using a low molecular mass calcium-binding nerve sheath protein named S100 along with calretinin to offer double immune labels for nerve bundles and ganglion cells.[12,13]

Unfortunately, IHC stains are costly and more complex than haematoxylin staining.[14] Hence, given the restriction of resources in government institutions, heath centres in Iraq still depend on HE in the diagnosis of HD.

The current study was planned to determine the accuracy of HE staining for the evaluation of ganglion cells and nerve bundle hypertrophy in comparison to calretinin and S100 IHC.

Materials and Methods

The retrospective study was conducted at the AL-Khansaa Teaching Hospital, Nineveh, Iraq, and comprised data from January 2017 to October 2020. After approval from the institutional ethics review committee, the sample was

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raised using randomly-sampled paraffinised blocks stored at the pathology laboratory. These blocks were from rectal suction biopsies (RSBs) performed on patients clinically suspected of HD. The archived blocks included had all the original HE-stained sections as well as the saved unstained slides. Blocks having lack of sufficient tissue or their stored unstained slides were excluded.

As part of institutional protocol related to histological sampling of rectal biopsies, 1-3 pieces of suction rectal biopsies are received, and the paraffin block is cut at 4µm thickness. Next, 5 sections per slide are prepared. The result is up to 75 haematoxylin-stained sections from each case before aganglionosis can be signed out.[15] In all cases, intervening unstained sections are stored at different levels for possible IHC analysis.

We prepared three slides from the stored unstained sections from each paraffin block, stained them with calretinin and S100 in a private pathology laboratory. The third slide of each sample was stained with HE to confirm the initial diagnosis and to act as the control. The diagnostic criteria of HD in HE sections are aganglionic specimens, which are often associated with hypertrophic submucosal nerve bundles.[16,17] The identification of ganglion cells in the submucosal plexus excludes HD[16]. The final HE diagnosis was compatible between the original slide and the current one.

Regarding IHC, the saved slides were prepared using the streptavidin-biotin method on formalin-fixed, paraffin-embedded tissue blocks sectioned (4µm), dewaxed, cleared and rehydrated. Endogenous peroxidase activity was inhibited by treating the slides with 3% hydrogen peroxide (H_2O_2) for 10min at room temperature, then washed with distilled water. The slides were incubated with the commercially available primary antibodies, including:Calretinin; Mouse Monoclonal antibody (Catalogue No. MA5-12539, clone DAK Calret 1, 1:10-1:20, Thermo Fisher Scientific, USA); and S100; Mouse Monoclonal Antibody (Catalogue No. MA5-12969, clone 4C4.9, 1:100, Thermo Fisher Scientific, USA)

The immune staining process was performed on Dako automated Auto-strainer Link 48 with Ultra Vision LP Large Volume Detection System HRP Polymer. Negative control slides were prepared by omitting the monoclonal antibodies from the staining protocol. Following immunostaining, the slides were examined for ganglion cells and intrinsic nerve fibres (INFs).

Calretinin had positively stained INFs in muscularis mucosa and lamina propria, as well as submucosal ganglion cells both nuclear and cytoplasmic in non-HD tissue, while in the HD-affected tissues, neither ganglion cells nor INFs were stained.[13]

For S100, the non-HD tissue showed few and weakly-stained INFs along with stained Schwann's cells surrounding negatively-stained ganglion cells. While in HD-affected tissue, the number and thickness of sub-mucosal nerve fibres were higher with more intense staining.[13] The maximum diameter of HE and S100 stained sub-mucosal nerve fibres were analysed using Digital Case Viewer software. Nerve fibres hypertrophies were interpreted as those with a diameter of $\geq$40µm.[16,17]

The histopathological and the IHC slides were blindly assessed by two expert pathologists, followed by a review for the agreement.

Data was analysed using SPSS 16. Histopathology was taken as the gold standard, and the studied markers were statistically analysed using Chi-square test. Descriptive statistics were used for age and gender. The sensitivity specificity, positive predictive value PPV), negative predictive value (NPV), accuracy, and kappa (κ) index were calculated.

Results

Of the 118 samples, 4(%) were excluded, and the final study sample comprised 114(%); 74(64.9%) males and 40(35.1%) females. The overall mean age was 32.51± 3.80 months (Table-1).

Features typical of HD, including the absence of ganglia with or without nerve bundle hypertrophy were identified by both pathologists with only one case discrepancy (p<0.001).

All biopsies were selected for calretinin IHC. Of them,

Table-1: Characteristics of patients with clinically suspected HD and final HE diagnosis in rectal suction biopsies.

Variables	Values	HD 28(24.6%)	Non-HD 86(75.4)
Gender			
Male	74 (64.9%)	21(75%)	53(61.6%)
Female	40 (35.1%)	7(25%)	33(38.4%)
Age (months)			
Mean±SD	32.51± 3.80	9.89± 1.04	35.71± 3.78
Median	12.50	9.00	22.00
Range	23 day- 144month	23day- 36month	1month- 144month
Age at RSB (months)			
Neonate	11 (9.6%)	6(21.4%)	5(5.8%)
Infant	46 (40.4%)	16(57.2%)	30(35%)
>12 months old	57(50%)	6(21.4%)	51(59.2%)
Total	114	28	86

HD: Hirschsprung disease, Non-HD: Non-Hirschsprung disease, HE: Haematoxylin- eosin, RSB: Rectal suction biopsy.

Table-2: Ganglion cells detection by HE (gold standard) staining and calretinin IHC staining in clinically suspected HD patients.

Accuracy parameters	HE	Calret	Superficial muscularis mucosae	Muscularis mucosa	Superficial submucosa	Submucosa	Total
TP	+	+	0	6	0	80	86
FN	+	_	0	0	0	1	01
FP	_	+	0	0	0	0	00
TN	_	_	0	2	0	25	27

TP: True positive, FP: False positive, FN: False negative, TN: True negative, HE: Haematoxylin-eosin, Calret: Calretinin, HD: Hirschsprung disease. The row-column association is statistically significant (p=0.0000).

Table-3: Nerve bundle hypertrophy detection by HE (gold standard) staining and S100 IHC staining in clinically suspected HD patients.

Accuracy parameters	HE	S100	superficial muscularis mucosae	Muscularis mucosa	Superficial submucosa	Submucosa	Total
TP	+	+	0	0	0	22	22
FP	_	+	0	0	0	0	00
FN	+	_	0	0	0	3	03
TN	_	_	0	0	18	71	89

TP: True positive, FN: False negative, FP: False positive, TN: True negative, HE: Haematoxylin-eosin, IHC: immunohistochemistry. The row/ column association is statistically significant (p< 0.0001).

Table-4: Correlation between HE staining and IHC staining (Calretinin and S100) in rectal suction biopsies of clinically suspected HD patients.

Variables	Ganglion cells N(%)		Nerve bundle hypertrophy N(%)	
	Present (non-HD)	Absent (HD)	Present (HD)	Absent (non-HD)
HE	86(75.4%)	28(24.6%)	25(21.9%)	89(78.1%)
Calret	87(76.3%)	27(23.7%)	-	-
S100	-	-	22(19.3%)	92(80.7%)

HD = Hirschsprung disease, Non-HD = Non-Hirschsprung disease, HE= Hematoxylin- eosin, HD: Hirschsprung disease, Calret = Calretinin. IHC: immunohistochemistry.

87(76.3%) cases revealed positive submucosal neurons associated with dark brown granular intrinsic nerve fibres in both lamina propria and mucosa. The immunostaining of neurons was present in both the cytoplasm and nuclei (Figure-1). In HD cases, the staining was consistently absent except for the mast cells that were used as positive calretinin internal control. Overall sensitivity, specificity, PPV, NPV and accuracy were 100%, 98.9%, 96.4%, 100%, and 99.1%, respectively (Table-2).

Based on histology, 28(24.6%) cases were negative for ganglion cells, and, of them 25(89.2%) revealed nerve bundle hypertrophy (Figure-2). The discrepancy between HE and S100 was in 3(2.6%) specimens. Overall sensitivity, specificity, PPV, NPV and accuracy were 100%, 96.7%, 88%, 100%, and 94.4% respectively (Table-3).

The correlation between HE staining and IHC staining obtained by the two pathologists led to final diagnosis using calretinin and the HE to be statistically near perfection (κ= 0.976, 95% confidence interval [CI]= 0.94-1.02, p=0.0001). Similarly, concordance of the detection of nerve bundle hypertrophy was statistically in strong agreement (κ= 0.923, 95% CI= 0.89-1.04. p<0.0001) (Table-4).

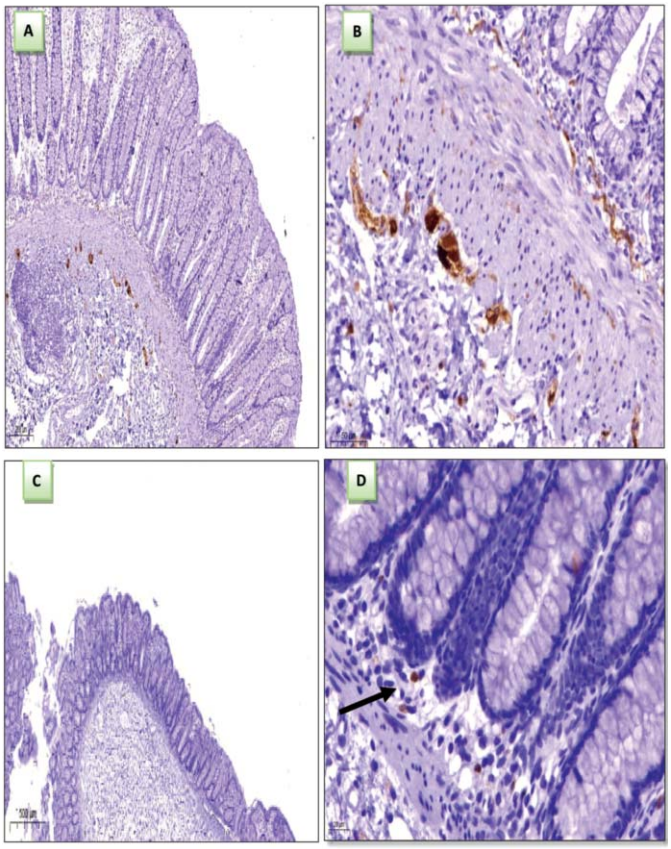

Figure-1: Calretinin immunohistochemistry (IHC) in rectal suction biopsies (RSBs). A: Calretinin expression in the mucosa and submucosa of non- Hirschsprung disease (HD) tissue (IHCx40). B: Granular immunostaining (nuclear and cytoplasmic) in the ganglion and intrinsic nerve fibres of the mucosa and submucosa of non-HD tissue (IHCx200). C: Complete absence of calretinin expression in the mucosa and submucosa of HD tissue (IHCx40). D: Negative immunostaining of ganglion cells and intrinsic nerve fibres in the mucosa and submucosa of HD tissue (IHCx400), positive calretinin internal control (mast cell arrow).

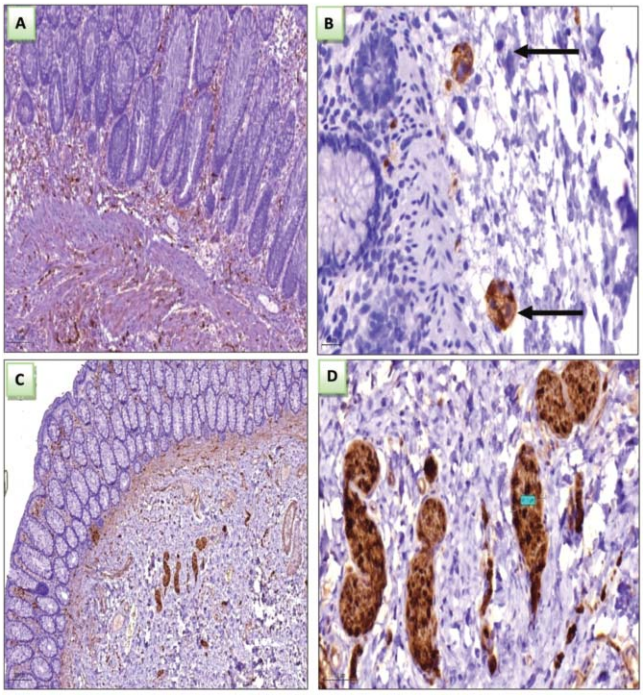

Figure-2: S100 immunohistochemistry (IHC) in rectal suction biopsies (RSBs). A-B: S100 highlighted intrinsic nerve fibres and negative ganglion cells (arrows), surrounded by positively-stained schwann cells in the submucosa of non-Hirschsprung disease (HD) tissue (x100 in A and x200 in B). C-D: Intense and prominent S100 positive expression showed hypertrophy of nerve fibres ≥40µm in the submucosa of HD tissue (IHCx40 in C, and x200 in D).

Discussion

Establishing the diagnosis of intestinal aganglionosis and forming the basis for surgical management depend on the evaluation of colonic and rectal biopsies for the presence or absence of ganglion cells.[18]

Despite the availability of various histochemical and IHC methods to raise and simplify the accuracy of ganglion cell identification, the histopathological examination of HE-stained serial sections remains the mainstay approach for the evaluation of clinically suspected HD.[18] However, the accuracy of routine stain depends on the balance between the two scales; the paediatric surgeon and the histopathologist. On one side, it requires the surgeon to provide adequate size, location and the number of rectal biopsy sections sent within 10% formalin solution. In many studies, the adequacy of an RSB was determined by at least two specimens of at least 3mm diameter obtained from the rectum at least 3cm above the dentate line.[19] On the other side, the pathologist should understand the microscopical findings of HD, decide whether it is an adequate biopsy or not, yield clear guidance to the technician when receiving such a specimen, and communicate with the surgeon when there is any diagnostic doubt. In most cases, this is enough to confirm or rule out HD.

In the current study, only one misdiagnosed HE-stained biopsy revealed calretinin immune expression, which belonged to a neonate without nerve hypertrophy. It was the same biopsy responsible for the disparity between the observers but, consequently, when calretinin was applied, both pathologists accurately diagnosed it. To the best of our knowledge, ganglion cells in this age group are small with dark nuclei, inconspicuous nucleoli, and surrounded by scanty cytoplasm, and, hence, mimic lymphocytes, endothelial cells, and are thus difficult to be diagnosed and missed on the routine stain.[19] In older kids, they are typically mature polygonal cells with round to oval nuclei, prominent nucleoli, and are surrounded by eosinophilic cytoplasm.[6] Therefore in this situation, calretinin helped lower the false-positive HD diagnosis in neonates eventually, reduced unnecessary bowel resection and pull-through.[10] In this case, we relied directly on the presence of calretinin positive small submucosal ganglion cell beside thin mucosal INFs as indirect evidence to rule out HD. The expression of calretinin in nerve fibres was observed in all ganglionic biopsies of clinically suspected HD and normal bowel. The observation has been mentioned in earlier studies.[3,20,21] The positively stained small nerve fibres in the lamina propria were valuable to exclude aganglionosis.[22] However, in a study, ganglion cells were only highlighted while the intrinsic nerve fibrils were negative.[23] Such a discrepancy shall not rule out an HD diagnosis, although this needs further investigation.[12] Calretinin staining was capable of recognising one specimen containing ganglia, which was missed by HE staining. Thus, according to the current study, the routine stain had the same sensitivity, but somewhat lower specificity than calretinin.[11] Other studies have also concluded that calretinin immunostaining was superior to HE staining as an ancillary method in diagnosing aganglionosis.[7,24]

The concordance of diagnosis of HD and non-HD using the gold standard and calretinin was statistically near perfection in the current study, which is in line with literature.[6,10]

In HD specimens, the loss of calretinin immunostain was in contrast to the gain of S100 expression.[13] S100 is a low-molecule calcium-binding protein.[25]

We noticed that S100 positive submucosal nerves in HD biopsies manifested two features. They markedly increased in size compared to intrinsic nerve fibres of the healthy ganglionic segments, and they increased in number. The last finding might be related to increasing tortuosity, leading to an increase in the numbers of transections of individual fibres in the tissue section. The first finding was

reinforced by morphometric analysis used in the study. Most HD specimens contain nerve bundles ≥40μm in diameter, while no fibre of that diameter was detected in non-HD specimens or healthy bowel segments.[12,20] Therefore, S100 staining of ≥40μm submucosal nerve bundles, a threshold for hypertrophic nerves, especially when they are multiple, is highly suggestive of intestinal aganglionosis.[12,13,20,24] Immunostaining intensity of S100 was different in the current study between non-HD and HD patients, which made it straightforward to interpret. Therefore, it could act as a supplement to increase the sensitivity of the gold standard method.[12]

The current study proved that HE remains the main and the standard stain to identify ganglion cells in an RSB of a patient suspected of HD. The histopathological HD diagnosis could be simple for experienced pathologists who frequently encounter such disorders, and when the quality of the tissue section is high-grade and the biopsy is adequate. In fact, with practice, even a small ganglion cell can be identified with confidence.[8] However, in rare circumstances, it may be desirable to use IHC to be definitive.[8]

Conclusion

The mainstay to confirm or exclude HD remains an accurate histopathological evaluation of the HE sections of an adequate colorectal biopsy.

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Conflict of Interest: None.

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References

1. Musa ZA, Qasim BJ, Ghazi HF, Shaikhly AW. Diagnostic roles of calretinin in hirschsprung disease: A comparison to neuron-specific enolase.Saudi J Gastroenterol. 2017; 23:60-6.
2. Schäppi M, Staiano A, Milla P, Smith V, Dias J, Heuschkel R, et al. A practical guide for the diagnosis of primary enteric nervous system disorders. J Pediatr Gastroenterol Nutr. 2013; 57:677-86.
3. Gonzalo DH, Plesec T. Hirschsprung Disease and Use of Calretinin in Inadequate Rectal Suction Biopsies. Arch Pathol Lab Med. 2013; 137:1099-102.
4. Kyrklund K, Sloots CEJ, de Blaauw I, Bjørnland K, Rolle U, Cavalieri D, et al. ERNICA guidelines for the management of rectosigmoid Hirschsprung's disease. Orphanet J Rare Dis. 2020; 15:164.
5. Anbardar MH, Geramizadeh B, Foroutan HR. Evaluation of Calretinin as a New Marker in the Diagnosis of Hirschsprung Disease.Iran J Pediatr. 2015; 25:e367.
6. Małdyk J, Rybczyńska J, Piotrowski D, Kozielski R. Evaluation of calretinin immunohistochemistry as an additional tool in confirming the diagnosis of Hirschsprung disease. Pol J Pathol. 2014; 65:34-9.
7. Rakhshani N, Araste M, Imanzade F, Panahi M, Tameshkel FS, Sohrabi MR, et al. Hirschsprung Disease Diagnosis: Calretinin Marker Role in Determining the Presence or Absence of Ganglion Cells. Iran J Pathol. 2016; 11:409-15.
8. Ambartsumyan L, Smith C, Kapur RP. Diagnosis of Hirschsprung Disease. Pediatr Dev Pathol. 2020; 23:8-22.
9. Kazemi Aghdam M, Khoddami M, Mollasharifi T, Almasi-Hashiani A. Diagnostic Value of Calretinin and S100 Immunohistochemistry in Hirschsprung's Disease. Int J Pediatr. 2019; 7:9577-89.
10. Mukhopadhyay B, Mukhopadhyay M, Mondal KC, Sengupta M, Paul A. Hirschsprung's Disease in Neonates with Special Reference to Calretinin Immunohistochemistry. JCDR. 2015; 9:EC06-9.
11. Sheir M, Samaka RM, Fakhry T, Albatanony AA. Comparative study between use of calretinin and synaptophysin immunostaining in diagnosis of Hirschsprung disease. Int Surg J. 2019; 6:658-63.
12. Jiang M, Li K, Li S, Yang L, Yang D, Zhang X, et al. Calretinin, S100 and protein gene product 9.5 immunostaining of rectal suction biopsies in the diagnosis of Hirschsprung'disease. Am J Transl Res. 2016; 8:3159.
13. Bachmann L, Besendörfer M, Carbon R, Lux P, Agaimy A, Hartmann A, et al. Immunohistochemical panel for the diagnosis of Hirschsprung's disease using antibodies to MAP2, calretinin, GLUT1 and S100. Histopathology. 2015; 66:824-35.
14. Serafini S, Santos MM, Tannuri ACA, Zerbini MCN, de Mendonça Coelho MC, de Oliveira Gonçalves J, et al. Is hematoxylin-eosin staining in rectal mucosal and submucosal biopsies still useful for the diagnosis of Hirschsprung disease? Diagn Pathol Open Access. 2017; 12:1-5.
15. Muise ED, Cowles RA. Rectal biopsy for Hirschsprung's disease: a review of techniques, pathology, and complications. World J Pediatr. 2016; 12:135-41.
16. Takawira C, D'Agostini S, Shenouda S, Persad R, Sergi C. Laboratory Procedures Update on Hirschsprung Disease. J Pediatr Gastroenterol Nutr. 2015; 60:598-605.
17. Kapur RP. Calretinin-Immunoreactive Mucosal Innervation in Very Short-Segment Hirschsprung Disease: A Potentially Misleading Observation. Pediatr Dev Pathol. 2014; 17:28-35.
18. Karim S, Hession C, Marconi S, Gang DL, Otis CN. The Identification of Ganglion Cells in Hirschsprung Disease by the Immunohistochemical Detection of ret Oncoprotein. Am J Clin Pathol. 2006; 126:49-54.
19. Rabah R. Total Colonic Aganglionosis: Case Report, Practical Diagnostic Approach and Pitfalls. Arch Pathol Lab Med. 2010; 134:1467-73.
20. Holland SK, Hessler RB, Reid-Nicholson MD, Ramalingam P, Lee JR. Utilization of peripherin and S-100 immunohistochemistry in the diagnosis of Hirschsprung disease. Mod Pathol. 2010; 23:1173-9.
21. Alexandrescu S, Rosenberg H, Tatevian N. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience. Int J Clin Exp Pathol. 2013; 6:2955-61.
22. Hiradfar M, Sharifi N, Khajedaluee M, Zabolinejad N, Jamshidi ST. Calretinin Immunohistochemistery: An Aid in the Diagnosis of Hirschsprung's Disease. Iran J Basic Med Sci. 2012; 15:1053-9.
23. Yang WI, Oh JT. Calretinin and microtubule-associated protein-2 (MAP-2) immunohistochemistry in the diagnosis of Hirschsprung's disease. J Pediatr Surg. 2013; 48:2112-7.
24. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease.J Clin Pathol Suppl Coll Pathol. 2004; 57:712-6.
25. Robey SS, Kuhajda FP, Yardley JH. Immunoperoxidase stains of ganglion cells and abnormal mucosal nerve proliferations in Hirschsprung's disease. Hum Pathol. 1988; 19:432-7