Section: Pathology

Original Research Article

CALRETININ IMMUNOHISTOCHEMISTRY STAINING PATTERN AND ITS HISTOPATHOLOGICAL CORRELATION IN THE DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE

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Received *: 06/06/2026*
Received in revised form *: 15/07/2026*
Accepted *: 31/07/2026*

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DOI: 10.70034/ijmedph.2026.3.321

Source of Support: Nil,
Conflict of Interest: None declared

Int J Med Pub Health
2026; 16 (3); 2015-2019

ABSTRACT

Background: Hematoxylin and Eosin (H&E) is the conventional method for diagnosing Hirschsprung's disease (HD), but its interpretation can be difficult and subject to interobserver variability, particularly for less experienced pathologists. Calretinin immunohistochemistry (IHC) has been proposed as a diagnostic adjunct. The aim is to compare the diagnostic sensitivity of Calretinin IHC with conventional H&E staining in suspected HD, to assess interobserver concordance between the two methods, and to characterise the staining pattern of ganglion cells and nerve fibres across bowel layers.

Materials and Methods: A retro-prospective study of 30 clinically suspected HD cases (22 full-thickness biopsies, 8 resected specimens; 68 paraffin blocks) was conducted at Coimbatore Medical College and Hospital. H&E and Calretinin IHC sections from ganglionic and aganglionic segments were independently evaluated by two blinded pathologists (one experienced, one in training) for the presence of ganglion cells and nerve bundle hypertrophy/immunoreactivity. Sensitivity, specificity, and Cohen's kappa were calculated.

Results: Of 30 cases, 5 were diagnosed as non-HD by both H&E and Calretinin IHC. H&E showed 87–90% sensitivity for identifying ganglion cells, with 13–23% equivocal readings depending on observer experience. Calretinin IHC eliminated equivocal readings entirely and confirmed a definitive diagnosis in all four additional cases left doubtful on H&E, improving diagnostic yield by 13.3%. Nerve fibre immunoreactivity by IHC had 88% sensitivity and 100% specificity for HD. Interobserver agreement was moderate for H&E (kappa = 0.574, 95% CI 0.36–0.78) but near-perfect for Calretinin IHC (kappa = 1.00).

Conclusion: Calretinin IHC is a useful adjunct to H&E in the diagnosis of HD, improving diagnostic sensitivity, resolving equivocal cases, and markedly reducing interobserver variability, particularly benefiting less experienced pathologists.

Keywords: Hirschsprung's disease; Calretinin; immunohistochemistry; ganglion cells; interobserver agreement.

INTRODUCTION

Hirschsprung's disease (HD), or aganglionic megacolon, is a congenital anomaly of innervation of the lower intestine, usually limited to the colon, resulting in partial or total functional obstruction.[1] The estimated incidence is 1 in 5000 live births.[2,3] HD arises from congenital absence of Meissner's and Auerbach's autonomic plexuses (aganglionosis) in the intestinal wall, most commonly limited to the distal colon (75% of cases), though the entire colon or bowel may be involved; the denervated segment is always contiguous. The etiology is thought to involve failure of neuroblast migration from the neural crest,

with a significant genetic component,at least 12 different genetic mutations and several syndromes have been associated with HD.

HD can be suspected clinically from presenting symptoms, contrast enema findings, and rectal manometry, but the gold standard for definitive diagnosis remains the demonstration of absent ganglion cells on histopathological examination of rectal biopsy.[4] The hallmark histological findings are aganglionosis and the presence of numerous hypertrophic cholinergic nerve fibres.[5,6] Diagnosis on H&E alone can be difficult, particularly in submucosal areas where ganglion cells are small and irregularly distributed, and in identifying immature ganglion cells in neonates. While H&E has approximately 95% diagnostic accuracy, the addition of immunohistochemical stains has been shown to raise sensitivity to as high as 99.7%.[7] Several ancillary technique,enzyme histochemistry (acetylcholinesterase, NADPH-diaphorase) and immunohistochemical markers such as neuron-specific enolase, Calretinin, c-kit, and S-100 have been evaluated to overcome these limitations. Acetylcholinesterase staining requires frozen sections and additional specimen, whereas Calretinin IHC has consistently shown superior diagnostic accuracy among IHC markers.

This study was undertaken to compare the diagnostic sensitivity of H&E and Calretinin IHC in suspected cases of HD, to characterise the staining pattern of ganglion cells and nerve fibres in the various layers of bowel tissue, and to evaluate interobserver concordance in diagnosis.

MATERIALS AND METHODS

Study design and setting: This retro-prospective study was conducted in the Department of Pathology, Coimbatore Medical College and Hospital, Coimbatore, from August 2016 to March 2018 (retrospective component: August–November 2016; prospective component: December 2016–March 2018), following approval from the Institutional Ethics Committee. A total of 30 clinically suspected cases of HD were analysed.

Selection criteria: All clinically suspected HD cases (age Day 1 to 15 years, either sex) who underwent rectal biopsy or bowel resection were included. Ill-fixed specimens, specimens not sent in formalin, and biopsies containing only anal canal epithelium or skeletal muscle were excluded.

Specimen processing: Twenty-two full-thickness biopsies and 8 resected specimens were received, formalin-fixed, and processed. One ganglionic and one aganglionic segment were sampled from each biopsy case (44 blocks); dilated ganglionic, spastic aganglionic, and transitional-zone tissue were sampled from resected specimens (24 blocks), giving a total of 68 paraffin blocks. Sections were stained with H&E and with Calretinin IHC (rabbit monoclonal anti-Calretinin, clone EP1798) using a standard indirect immunoperoxidase method with heat-induced antigen retrieval, DAB chromogen, and haematoxylin counterstain.

Interpretation: H&E slides from all 68 blocks were independently examined by two pathologists — an experienced observer (Observer 2) and a less-experienced observer (Observer 1), blinded to each other's findings for the presence or absence of ganglion cells and hypertrophic nerve bundles in the submucosa and muscularis propria. Findings were recorded as positive, negative, or equivocal. Non-HD (NHD) was defined by identification of at least one ganglion cell on representative sections; absence of ganglion cells, with or without hypertrophic nerve fibres, was considered diagnostic of HD.

The same 68 blocks were subsequently stained with Calretinin IHC and evaluated independently by the same two observers, blinded to the H&E diagnosis, for ganglion cell and nerve fibre immunoreactivity across the lamina propria, muscularis mucosa, submucosa, and muscularis propria. Calretinin positivity was defined as (i) intense, granular, linear staining of nerve fibres, or (ii) diffuse, strong cytoplasmic and nuclear staining of ganglion cells and supporting Schwann cells. Nonspecific staining of scattered mast cells served as an internal positive control. Staining intensity in ganglion cells was semi-quantitatively graded 0 (absent) to 3 (strong), and neural plexus reactivity was scored on an analogous scale.[8]

Statistical analysis: Data were analysed using SPSS version 28 for Windows. Descriptive data were expressed as mean ± standard deviation or as number and percentage. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value of Calretinin IHC relative to H&E were calculated using Observer 2's data. Cohen's kappa coefficient (9) was used to assess interobserver agreement for both H&E and Calretinin IHC. A p-value < 0.05 was considered statistically significant.

RESULTS

Of the 30 patients studied, 22 (73.3%) were male and 8 (26.7%) female (male:female ratio 2.75:1). Fifteen (50%) presented before 1 month of age, 11 (36.6%) between 1 month and 1 year, and 4 (13.4%) after 1 year. Classic short-segment HD (SHD) accounted for 26 cases (86.7%), long-segment HD (LHD) for 3 (10%), and total colonic aganglionosis (TCA) for 1 (3.3%). Twenty-two patients (73.3%) were diagnosed on full-thickness rectal/colonic biopsy and 8 (26.7%) on resection specimens.

On H&E, ganglion cells were identified in 4/30 (13.3%) submucosal and 5/27 (18.5%) muscularis propria samples of the clinically aganglionic segment, resulting in these five cases being reclassified as non-HD. Hypertrophic nerve bundles were present in 63–70% of the remaining aganglionic samples. Hypertrophic nerve bundle assessment showed 86.4% sensitivity, 100% specificity, and

100% PPV for HD. Nerve bundle hypertrophy was less frequent in long-segment disease (50%) than in short-segment disease (69–74%), consistent with prior reports.[10,11]

On Calretinin IHC, the same five non-HD cases showed ganglion cell and nerve fibre immunoreactivity. Of the remaining 25 cases diagnosed as HD by ganglion cell negativity, 3 (12%) showed faint, punctate nerve fibre immunoreactivity distinctly different from the confluent, dark, granular pattern seen in non-HD cases — a false-positive pattern also described by Hiradfar et al,[11] and Kapur et al.[15] Nerve fibre immunoreactivity as a diagnostic criterion had a sensitivity of 88% and specificity of 100%.

Table 1: Demographic and clinical profile of the study population (N = 30)

Variable	Category	n (%)
Age	< 1 month	15 (50.0)
	1 month – 1 year	11 (36.6)
	> 1 year	4 (13.4)
Gender	Male	22 (73.3)
	Female	8 (26.7)
Extent of disease	Short-segment HD	26 (86.7)
	Long-segment HD	3 (10.0)
	Total colonic aganglionosis	1 (3.3)
Specimen type	Full-thickness biopsy	22 (73.3)
	Resection	8 (26.7)

Table 2: Ganglion cell and nerve fibre findings on H&E and Calretinin IHC (aganglionic segment, Observer 2)

Bowel layer	Ganglion cells positive (H&E)	Ganglion cells positive (IHC)	Nerve fibre reactivity (IHC)
Submucosa	4 (13.3%)	5 (16.7%)	8 (26.7%)
Muscularis propria	5 (18.5%)	5 (18.5%)	8 (29.6%)

Equivocal readings on H&E were substantially more frequent for the less-experienced Observer 1 (23%) than for Observer 2 (5–10%); Calretinin IHC yielded no equivocal readings for either observer. Sensitivity and PPV of H&E for ganglion cell detection ranged from 87–100% across observers and layers, but with reduced diagnostic confidence due to equivocal calls.

Table 3: Diagnostic performance and interobserver agreement

Parameter	H&E	Calretinin IHC
Sensitivity (ganglion cell / nerve fibre detection)	87–90%	88–90%
Specificity	100%	100%
Equivocal readings (Observer 1)	23%	0%
Equivocal readings (Observer 2)	5–10%	0%
Interobserver agreement (Cohen's kappa)	0.574 (moderate)	1.00 (near-perfect)

Based on final independent diagnoses, both observers classified 5 cases (16.7%) as non-HD and the remainder as HD or suspicious using H&E; Observer 1 reported 4 suspicious cases (13.3%) and Observer 2 reported 1 (3.3%) on H&E. Calretinin IHC resolved every suspicious H&E case as HD for both observers, increasing the proportion of cases diagnosed as HD from 70–80% (H&E) to 83.3% (IHC), a 13.3% improvement in diagnostic yield, concordant with findings reported by Guinard-Samuel et al. (16) and Kannaiyan et al. (10). Among ganglionic-segment samples with identifiable ganglion cells (n = 26), Calretinin reactivity was graded 3+ (strong) in 19 (73%) and 2+ (moderate) in 7 (27%).

Table 4: Final diagnosis by H&E and Calretinin IHC (N = 30)

Diagnosis	H&E – Observer 1	H&E – Observer 2	Calretinin IHC (both observers)
HD	21 (70.0%)	24 (80.0%)	25 (83.3%)
Non-HD	5 (16.7%)	5 (16.7%)	5 (16.7%)
Suspicious/equivocal	4 (13.3%)	1 (3.3%)	0 (0%)

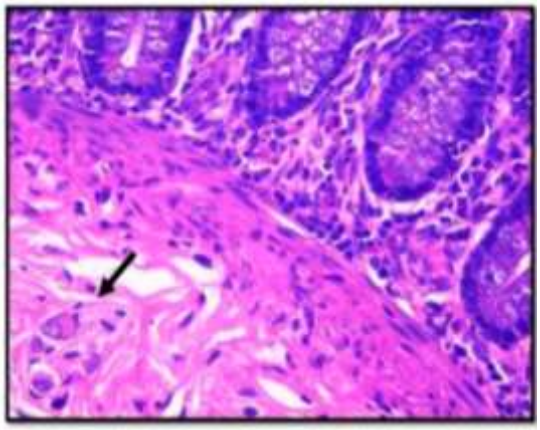

Fig 1:H & E section shows submucosal-Meissner's Plexus (arrow)(400X)

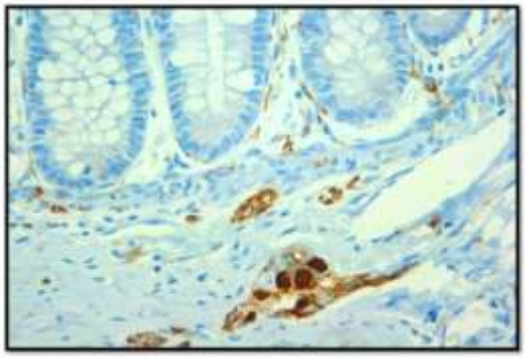

Fig 2:Calretinin IHC section shows immunoreactivity in the submucosal plexus (400X)

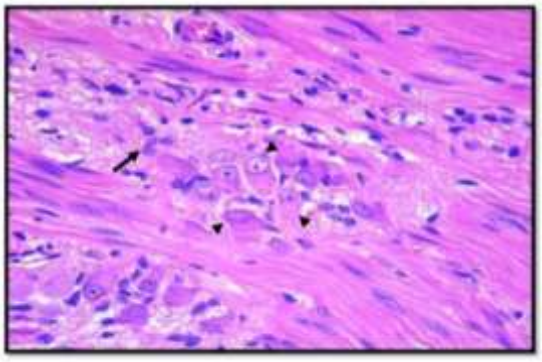

Fig 3:H &E section shows Ganglion cells (neuronal cell bodies) characterized by a large polygonal cells with abundant pink cytoplasm and eccentric nucleus ,prominent nucleoli (arrowhead),spindle neural projections and Shwann cells (arrow)are also intermixed (400X)

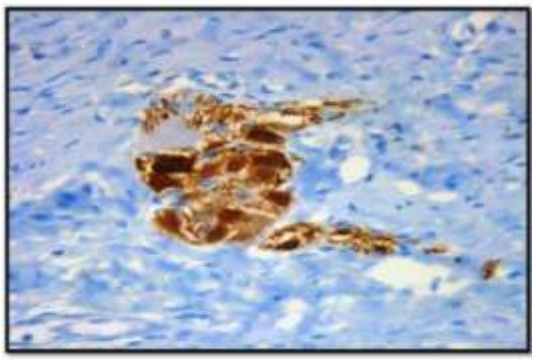

Fig 4:Calretinin IHC section shows strong reactivity to ganglion cells and moderate reactivity to nerve plexus (400X)

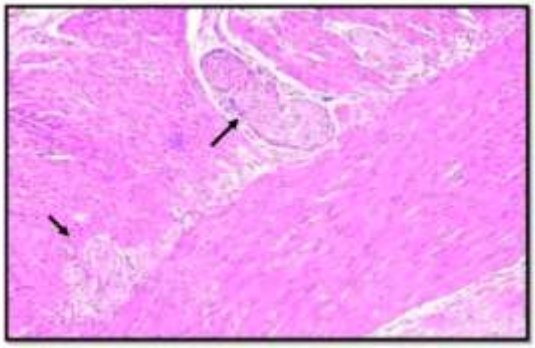

Fig 5:H & E section from a HD case shows Hypertrophic myenteric nerve plexus (arrows) lacking ganglion cells (100X)

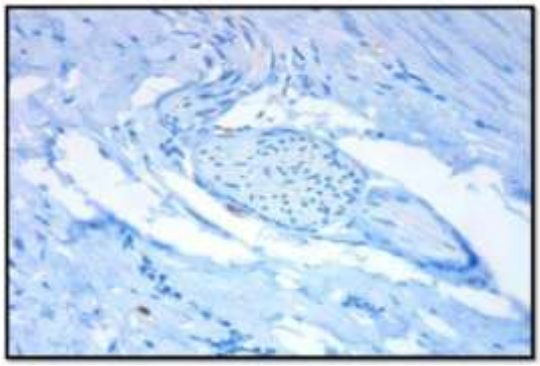

Fig 6: Calretinin IHC section from HD case shows submucosal hypertrophic nerve trunk with no immunoreactivity for ganglion cells and nerve fibres cells (400X)

DISCUSSION

In this series of 30 suspected HD cases, the median age at presentation and male preponderance (2.75:1) were consistent with earlier Indian and international series,[10,11] though slightly lower than the classically reported ratio of 4:1,[12,13] likely reflecting the smaller sample size. The proportion of short-segment disease (86.7%) closely matched the distribution reported by Hiradfar et al,[11] while the incidence of TCA (3.3%) was lower than that reported by Kannaiyan et al,[10] again probably a sample-size effect.

Two H&E features were assessed: absence of ganglion cells (the pathognomonic finding) and nerve bundle hypertrophy, a useful secondary feature. Hypertrophic nerve bundles showed 86.4% sensitivity, 100% specificity, and 100% PPV for HD, similar to the pattern described by Narayanan et al,[14] who found nerve bundle hypertrophy less consistently present in extended-segment disease.

Calretinin IHC replicated the H&E diagnosis of non-HD in all five cases and additionally showed nerve fibre immunoreactivity with 88% sensitivity and 100% specificity. Faint, punctate nerve fibre positivity was noted in 12% of otherwise HD-negative cases, a pattern also reported by Hiradfar et al,[11] in 6.7% of cases and by Kapur et al,[15] who could not fully explain the finding; Guinard-Samuel et al,[16] suggested it may mark the beginning of the transition zone.

The principal advantage of Calretinin IHC over H&E was the elimination of equivocal diagnoses. Observer 1 (less experienced) recorded equivocal findings in 23% of H&E readings versus only 5–10% for Observer 2, but neither observer had any equivocal readings with Calretinin IHC. This translated into a 13.3% improvement in diagnostic yield with IHC, matching findings from Guinard-Samuel et al., where 12 of 131 H&E-equivocal cases were resolved with Calretinin,[16] and Kannaiyan et al., where all 15 H&E-equivocal cases were correctly diagnosed with Calretinin.[10]

Interobserver agreement showed the clearest benefit of Calretinin IHC: kappa improved from 0.574 (moderate agreement) with H&E to 1.00 (perfect agreement) with IHC. This is comparable to the near-perfect agreement (kappa = 0.97) reported by Anbardar et al. across 82 paraffin blocks,[17] and supports Calretinin IHC as a particularly valuable adjunct in settings where less-experienced pathologists are involved in HD diagnosis.

CONCLUSION

Calretinin immunohistochemistry is a useful adjunct to H&E in the diagnosis of Hirschsprung's disease, improving diagnostic sensitivity, resolving equivocal cases, and reducing interobserver variability particularly benefiting centres or pathologists with limited experience in HD reporting. The additional cost of Calretinin IHC is justified by the gains in diagnostic accuracy and the potential to spare patients the morbidity and expense of repeat or second-look surgery. The 12% false-positive nerve fibre immunoreactivity observed in confirmed HD cases, though qualitatively distinct from the normal staining pattern, warrants further study.

REFERENCES

1. Heanue TA, Pachnis V. Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nat Rev Neurosci. 2007 Jun;8(6):466-79.
2. Spouge D, Baird PA. Hirschsprung disease in a large birth cohort. Teratology. 1985;32:171–7.
3. Suita S, Taguchi T, Kamimura T, et al. A changing profile of Hirschsprung's disease in Japan — result from a nationwide survey. J Jap Soc Pediatr Surg. 1996;32:952–65.
4. Dobbins WO, Bill AH Jr. Diagnosis of Hirschsprung disease excluded by rectal suction biopsy. N Engl J Med. 1965;272:990–993.
5. Jay V. Legacy of Harald Hirschsprung. Pediatr Dev Pathol. 2001;4:203–4.
6. Swenson O, Rheinlander HF, Diamond I. Hirschsprung's disease; a new concept of the etiology; operative results in 34 patients. N Engl J Med. 1949;241:551–6.
7. Harjai MM. Hirschsprung's disease: revisited. J Postgrad Med. 2000;46:52.
8. Zuikova V, Franckevica I, Strumfa I, Melderis I. Immunohistochemical diagnosis of Hirschsprung's disease and allied disorders. Acta Chirurgica Latviensis. 2015;15:50-7.
9. K. Cohen J. A coefficient of agreement for nominal scales. Educ Psychol Meas. 1960;20:37–46.
10. Kannaiyan L, Madabhushi S, Malleboyina R, Are NK, Reddy KR, Rao B. Calretinin immunohistochemistry: a new cost-effective and easy method for diagnosis of Hirschsprung's disease. J Indian Assoc Pediatr Surg. 2013;18:66-8.
11. Hiradfar M, Sharifi N, Khajedaluee M, Zabolinejad N, Taraz Jamshidi S. Calretinin immunohistochemistry: an aid in the diagnosis of Hirschsprung's disease. Iran J Basic Med Sci. 2012;15(5):1053.
12. Torfs C. An epidemiological study of Hirschsprung's disease in a multiracial California population. Proceedings of the Third International Meeting: Hirschsprung's Disease and Related Neurocristopathies. Evian, France; 1998.
13. Russell MB, Russell CA, Niebuhr E. An epidemiological study of Hirschsprung's disease and additional anomalies. Acta Paediatr. 1994;83:68–71.
14. Narayanan SK, Soundappan SS, Kwan E, Cohen RC, Charlton A, Cass DT. Aganglionosis with the absence of hypertrophied nerve fibres predicts disease proximal to rectosigmoid colon. Pediatr Surg Int. 2016;32:221–226.
15. Kapur RP, Reed RC, Finn LS, Patterson K, Johanson J, Rutledge JC. Calretinin immunohistochemistry versus acetylcholinesterase histochemistry in the evaluation of suction rectal biopsies for Hirschsprung disease. Pediatr Dev Pathol. 2009 Jan-Feb;12:6-15.
16. Guinard-Samuel V, Bonnard A, De Lagausie P, Philippe-Chomette P, Alberti C, El Ghoneimi A, et al. Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease. Mod Pathol. 2009;22:1379-1384.
17. Anbardar MH, Geramizadeh B, Foroutan HR. Evaluation of Calretinin as a new marker in the diagnosis of Hirschsprung disease. Iran J Pediatr. 2015 April;25(2):e367.