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Comparison of calretinin and S100 as immunohistochemical markers for diagnosing hirschsprung's disease

Perbandingan calretinin dengan S100 sebagai marker immunohistokimia dalam menegakkan diagnosa hirschsprung disease

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Abstract

Background: : Hirschsprung's disease is a congenital disorder caused by the failure of neural crest cell migration, resulting in the absence of ganglion cells in the myenteric and submucosal plexuses. Histopathological examination with hematoxylin-eosin (H&E) staining is the gold standard, but its interpretation is often challenging. Immunohistochemical markers such as Calretinin and S100 have been developed to improve diagnostic accuracy.
Objective: To compare the diagnostic value of Calretinin and S100 as immunohistochemical markers in establishing the diagnosis of Hirschsprung's disease. Methods: An observational analytical study with a cross-sectional design using 33 samples of patients with Hirschsprung's disease who underwent biopsies at H. Adam Malik General Hospital, Medan, from 2022 to 2025. using McNemar's exact test. Statistical significance was set at $p < 0.05$. The reference diagnosis was established based on histopathological evaluation of H&E staining in correlation with clinical findings and surgical outcomes, when available. Cases were classified as Hirschsprung's disease if histological examination demonstrated the absence of ganglion cells, consistent with the clinical presentation of functional intestinal obstruction.
Results: H&E + Calretinin examination showed positive results for Hirschsprung's disease in 81.8% of the cases, while H&E + S100 showed positive results in 78.8% of the cases ($p = 0.002$). The sensitivity and specificity of calretinin were 96.2% (95% CI: 81.0-99.9) and 83.3% (95% CI: 35.9-99.6) with an overall accuracy of 93.9%, whereas those of S100 were 96.1 % (95% CI: 75.7–99.1) and 71.4 % (95% CI: 35.9–99.6), respectively. with an overall accuracy of 90.9%. McNemar's exact test revealed no statistically significant difference between Calretinin and S100 ($p = 1.000$)
Conclusion: Calretinin has a higher diagnostic value than S100 in detecting Hirschsprung's disease, with better sensitivity and specificity. Although Calretinin showed slightly higher sensitivity, no statistically significant difference was observed between the two markers. Therefore, both markers may serve as reliable adjunct immunohistochemical tools to support the histopathological diagnosis of Hirschsprung's disease.

Keywords:
Calretinin, diagnosis, immunohistochemistry, Hirschsprung's disease, S100

Abstrak

Latar Belakang: Penyakit Hirschsprung adalah kelainan kongenital yang disebabkan oleh kegagalan migrasi sel krista neural, sehingga terjadi ketiadaan sel ganglion pada pleksus mienterikus dan submukosa. Pemeriksaan histopatologi dengan pewarnaan hematoksilin–eosin (H&E) merupakan baku emas, namun interpretasinya seringkali sulit. Penanda imunohistokimia seperti calretinin dan S100 dikembangkan untuk meningkatkan akurasi diagnosis.
Tujuan: Membandingkan nilai diagnostik calretinin dan S100 sebagai penanda imunohistokimia dalam menegakkan diagnosis penyakit Hirschsprung.
Metode: Penelitian analitik observasional dengan desain potong lintang menggunakan 33 sampel pasien penyakit Hirschsprung yang menjalani biopsi di RSUP H. Adam Malik Medan pada periode 2022–2025. Analisis dilakukan menggunakan uji McNemar exact. Nilai $p < 0,05$ dianggap bermakna secara statistik. Diagnosis rujukan ditetapkan

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berdasarkan evaluasi histopatologi pewarnaan H&E yang dikorelasikan dengan temuan klinis dan luaran pembedahan bila tersedia. Kasus diklasifikasikan sebagai penyakit Hirschsprung apabila pemeriksaan histologis menunjukkan tidak ditemukannya sel ganglion, sesuai dengan gambaran klinis obstruksi usus fungsional.
Hasil: Pemeriksaan H&E + Calretinin menunjukkan hasil positif untuk penyakit Hirschsprung sebesar 81,8%, sedangkan H&E + S100 sebesar 78,8% (p = 0,002). Sensitivitas dan spesifisitas calretinin masing–masing adalah 96,2% (IK 95%: 81,0–99,9) dan 83,3% (IK 95%: 35,9–99,6) dengan akurasi keseluruhan 93,9%. Sementara itu, S100 memiliki sensitivitas 96,1% (IK 95%: 75,7–99,1) dan spesifisitas 71,4% (IK 95%: 35,9–99,6) dengan akurasi keseluruhan 90,9%. Uji McNemar exact menunjukkan tidak terdapat perbedaan yang bermakna secara statistik antara calretinin dan S100 (p = 1,000).
Kesimpulan: Calretinin memiliki nilai diagnostik yang lebih tinggi dibandingkan S100 dalam mendeteksi penyakit Hirschsprung, dengan sensitivitas dan spesifisitas yang lebih baik. Meskipun calretinin menunjukkan sensitivitas yang sedikit lebih tinggi, tidak ditemukan perbedaan yang bermakna secara statistik antara kedua penanda tersebut. Oleh karena itu, kedua penanda dapat digunakan sebagai alat imunohistokimia tambahan yang andal dalam mendukung diagnosis histopatologis penyakit Hirschsprung.

Kata Kunci:
Calretinin, diagnosis, imunohistokimia, penyakit Hirschsprung, S100

Introduction

Hirschsprung disease is a congenital condition that contributes significantly to the mortality rate of newborns and children under five years of age. Hirschsprung disease causes intestinal problems in infants. One common symptom of Hirschsprung's disease in infants is the inability to have a bowel movement within 24–48 h of birth. In infants, symptoms include chronic constipation, abdominal distension, and growth retardation. Hirschsprung's disease is most common in newborns, with an estimated global incidence of 1 in 5,000 live births, and is more common in males (Sergi et al., 2017).

Hirschsprung disease (HD), a disorder of the enteric nervous system (ENS), is a rare congenital developmental disorder of the gastrointestinal tract. It is characterized by the failure of vagal-derived enteric neural crest (NC) cells (neurocrystopathy) to migrate fully craniocaudally during embryonic development and colonize the entire intestine, leaving aganglionic segments of varying lengths (Sergi et al., 2017).

The most prominent etiopathogenetic factor in the development of Hirschsprung's disease is NC disorder, a condition classified as a neurocritopathy. The absence of ENS ganglia in the affected portion of the intestine causes functional obstruction, which manifests clinically soon after birth. (Bradnock et al., 2017; Montalva et al., 2023).

The diagnosis of Hirschsprung's disease is based on a combination of clinical features, radiological findings, and histopathological evaluation of biopsy samples. Histopathological examination of rectal biopsies confirms the diagnosis by highlighting the association between the absence of ganglion cells (GCs) in the submucosal and myenteric plexuses and hypertrophy of nerve fibers in the aganglionic segment (Miles et al., 2023). Conventional histopathological processing with hematoxylin and eosin (H&E) staining is often limited, especially when the submucosa is sparse or ganglion cells are immature, making visualization difficult. This necessitates the use of immunohistochemical (IHC) methods, such as calretinin and S100, to enhance diagnostic accuracy (Beltman et al., 2022).

Many institutions use hematoxylin and eosin histopathology examinations combined with IHC calretinin and S100 to help diagnose Hirschsprung's disease (Beltman et al., 2022; Kazemi Aghdam et al., 2019). This study aimed to compare these two IHC examinations in diagnosing Hirschsprung's disease by assessing which is better in terms of sensitivity and specificity. The examinations being compared were Hematoxylin Eosin (HE) staining with calretinin or Hematoxylin Eosin (HE) with S100. Therefore, it can be considered that only one examination is used, considering cost-effectiveness without reducing the accuracy of the diagnosis of patients with Hirschsprung's disease. Based on the above background, the researcher is interested in comparing the diagnostic value of calretinin with S100 as an immunohistochemical marker in diagnosing Hirschsprung's Disease at Adam Malik Hospital, Medan (Ambartsumyan et al., 2020).

Methods

This observational analytical study with a cross-sectional design aimed to compare the diagnostic

value of calretinin and S100 immunohistochemical examinations in establishing the diagnosis of Hirschsprung's disease. The study was conducted at the Department of Surgery and Anatomical Pathology Laboratory, Faculty of Medicine, University of North Sumatra/H. Adam Malik General Hospital, Medan, from January to June 2025.

The study population included all pediatric patients who underwent rectal biopsy for suspected Hirschsprung disease between 2022 and 2025. Samples were selected using the consecutive sampling method, that is, all patients who met the inclusion criteria until the minimum number was met. The inclusion criteria were patients aged less than 18 years and having available histopathology preparations, while the exclusion criteria were patients with a history of necrotizing enterocolitis (NEC) related to Hirschsprung's disease. Based on the calculation of the sample size using the proportion formula, a minimum of 33 samples was obtained. The reference diagnosis was established based on histopathological evaluation of H&E staining in correlation with clinical findings and surgical outcomes, when available. Cases were classified as Hirschsprung's disease if histological examination demonstrated the absence of ganglion cells, consistent with the clinical presentation of functional intestinal obstruction.

The independent variable in this study was the result of the H&E examination combined with calretinin and S100 staining, while the dependent variable was the diagnosis. Cases were classified as Hirschsprung's disease if histological examination demonstrated the absence of ganglion cells, consistent with the clinical presentation of functional intestinal obstruction. Hirschsprung's disease is based on the presence or absence of ganglion cells. Secondary data were collected from medical records and laboratory results and sorted based on the research variables. Paraffin blocks were serially sectioned (2–4 µm), mounted on glass slides, and subjected to hematoxylin and eosin staining and immunohistochemistry using primary antibodies against calretinin and S100 at a dilution of 1:100. The immunohistochemistry procedure included deparaffinization, rehydration, antigen retrieval at 98°C, inactivation of endogenous peroxidase, overnight incubation with primary antibodies at 40°C, administration of secondary antibodies, 3,3'-diaminobenzidine (DAB) staining, and hematoxylin counterstaining. Calretinin and S100 expression was assessed semiquantitatively by the researcher and two anatomic pathologists, with categories of negative, weak, or strong.

Immunohistochemical staining for calretinin and S100 was independently evaluated by two experienced pathologists blinded to the H&E results. For calretinin, positive staining was defined as the presence of cytoplasmic and/or nuclear staining in intrinsic nerve fibers or ganglion cells, whereas the complete absence of staining was classified as negative. For S100, positive staining was defined as the presence of nerve fiber staining in the submucosal and/or myenteric plexus, while the absence of staining was considered negative.

Statistical analyses were performed using SPSS version 26. Diagnostic parameters, including sensitivity, specificity, positive predictive value, negative predictive value, and accuracy, were calculated using 2×2 contingency tables with H&E staining as the reference standard. Ninety-five percent confidence intervals (95% CI) were calculated for sensitivity and specificity using the exact (Clopper–Pearson) method. Because the same biopsy specimens underwent both calretinin and S100 staining, the diagnostic results were considered as paired data. Therefore, a comparison between the two markers was performed using McNemar's test, which is appropriate for paired categorical data. Statistical significance was set at $p < 0.05$.

This study was reviewed and approved by the Chair of the Health Research Ethics Committee of Universitas Sumatera Utara (No. 1399/KEPK/USU/2025) following the Neuremberg Code and the Declaration of Helsinki.

Results

Table 1 shows that the majority of patients in this study were male (64%) and were under 1 year of age (53.1%). This finding aligns with the epidemiology of Hirschsprung disease, which is more common in male neonates.

Table 2 shows that most patients with Hirschsprung's disease had H&E + calretinin results (81.8%) and H&E + S100 results (78.8%). A p-value <0.05 indicated a significant difference in both

calretinin and S100 immunohistochemical staining examinations.

Table 1. Frequency distribution of the demographic characteristics of the study.

Variables	f	%
Gender		
Male	21	64
Female	12	36
Age		
<1 year	18	54.5
1-5 years	9	27.3
>5 years	6	18.2

Table 2. Distribution of immunohistochemical staining results of calretinin and S100

Inspection	Hirschsprung's disease (n, %)	Non-Hirschsprung's disease (n, %)
H&E + Calretinin	27 (81.8%)	6 (18.2%)
H&E + S100	26 (78.8%)	7 (21.2%)

Table 3 shows that the sensitivity and specificity of calretinin were 96.2% (95% CI: 81.0–99.9) and 83.3% (95% CI: 35.9–99.6), respectively, with an overall accuracy of 93.9 %, whereas those of S100 were 96.1% (95% CI: 75.7 – 99.1) and 71.4% (95% CI: 35.9–99.6), respectively. with an overall accuracy of 90.9%, which means that the relationship between the examination results and the diagnosis of Hirschsprung's disease is statistically significant. As the same specimens underwent both immunohistochemical staining procedures, McNemar's test was used to compare the paired diagnostic results. No statistically significant difference was observed between calretinin and S100 expression (p = 1.000).

Table 3. Comparative analysis of calretinin and S100 immunohistochemical staining diagnostics

Inspection	Sensitivity (%)	Specificity (%)
H&E + Calretinin	96.2	83.3
H&E + S100	96.1	71.4

Discussion

Hirschsprung's disease is a congenital disorder characterized by the absence of ganglion cells in the myenteric and submucosal plexuses, resulting in functional intestinal obstruction.[6] This condition results from the failure of neural crest cell migration during embryonic development, resulting in a non-functioning segment of the intestine. Clinically, the disease appears in the neonatal period with symptoms ranging from delayed meconium passage and abdominal distension to chronic constipation 0. Early diagnosis is crucial to avoid serious complications, such as Hirschsprung's-associated enterocolitis.

The diagnosis of Hirschsprung's disease has traditionally relied on a combination of clinical, radiological, and histopathological findings (Ambartsumyan et al., 2020). Histopathological examination with hematoxylin-eosin (H&E) staining to detect the presence or absence of ganglion cells is the gold standard. However, with the development of immunohistochemical techniques, markers such as S100 and calretinin have been proposed as additional methods to improve diagnostic accuracy, especially in cases where H&E interpretation is difficult (Kazemi Aghdam et al., 2019).

S100 staining marks nerve fiber hypertrophy, whereas calretinin identifies the presence of ganglionic nerve fibers. Previous studies have shown varying results regarding the sensitivity and specificity of these two markers. Therefore, this study was conducted to compare the diagnostic value of histopathology, S100 immunohistochemistry (IHC), and calretinin in patients with Hirschsprung's disease at H. Adam Malik General Hospital, Medan, Indonesia. The results of this study are expected to contribute to more efficient, accurate, and cost-effective diagnostic methods for Hirschsprung's disease, while also comparing local results with existing global reports.

This finding is consistent with the study by Palissei et al. (2021) which reported that HD is more common in males with a ratio of 4:1 (Palissei et al., 2021), and the report by Hei et al. (2022) which indicated the most common age of presentation in neonates (Hei Ha et al., 2022)

However, these results contradict those of the present study, which found a higher incidence in females with Hirschsprung's disease associated with Down syndrome. This difference is likely due to the different study populations; in this study, most patients did not have specific genetic comorbidities, such as Down syndrome. The male predominance and neonatal age support the understanding that genetic factors and

embryological development play a major role in the pathogenesis of Hirschsprung's disease, consistent with the theory of imperfect neural crest cell migration (Klein & Varga, 2020; Yu et al., 2021)

These results are in line with those of Alexandrescu, Rosenberg, and Tatevian (2013) and Beltman et al.., which also supports these results, where negative calretinin is a strong indicator of the presence of an aganglionic segment (Alexandrescu et al., 2013; Beltman et al., 2022). Table 3 shows that the calretinin IHC examination had a sensitivity of 96.2% and a specificity of 83.3%. The S100 IHC examination had a sensitivity of 96.1% and specificity of 71.4%. These results are in line with the study of Kazemi Aghdam et al. (2019), which showed that immunohistochemical examination (IHC) using calretinin had 100% sensitivity and specificity in detecting Hirschsprung's disease, while S100 showed 61.9% sensitivity and 93% specificity in detecting Hirschsprung's disease (Kazemi Aghdam et al., 2019).

However, these results differ from those reported by Das and Mohanty (2017), who reported a high level of misinterpretation of calretinin in premature neonates, leading to false-positive results. This may be explained by the fact that, in this study, most patients were full-term neonates with mature submucosal tissue, making negative calretinin results more consistent and valid. Therefore, the results of S100 IHC and calretinin examinations can be considered very helpful in diagnosis, but variability based on age and biopsy tissue thickness must still be considered (Das & Mohanty, 2017).

In contrast, Heuckeroth (2018) reported that calretinin sensitivity can decrease by up to 85% in cases with immature ganglia, which poses a risk of misinterpretation. This discrepancy may be due to factors such as variations in staining techniques, examiner experience, and the quality of the biopsy specimens, as rigorous quality control was maintained in this study by the anatomical pathology laboratory. The small discrepancy between this study and several others suggests that while IHC is very useful, H&E histopathology remains indispensable as a basis for a definitive diagnosis (Heuckeroth, 2018).

The limitations of this study include the relatively small sample size and retrospective nature. Furthermore, potential bias in CPI interpretation between examiners is still possible, even though evaluations were performed by more than one pathologist. Not all variations in IHC techniques (such as different types of calretinin antibodies) could be evaluated. Further studies with prospective, multicenter designs and larger sample sizes are needed to confirm these findings. The relatively small sample size (n = 33) may have limited the statistical power to detect small differences between the two markers in the study. With only six non-Hirschsprung cases, the estimates of specificity and confidence intervals were relatively wide. Therefore, larger multicenter studies are needed to confirm these findings and improve the precision of diagnostic estimates.

Hirschsprung’s disease is conventionally diagnosed through histopathological examination with hematoxylin-eosin (HE) staining to confirm the absence of ganglion cells and nerve fiber changes. However, certain cases with H&E staining alone may yield questionable findings because of technical limitations, suboptimal tissue orientation, or subtle morphological changes. In such situations, IHC examination with S100 and calretinin markers may provide meaningful and additional information.

S100 protein is a Schwann cell marker that facilitates the visualization of nerve fibers and allows for the assessment of their distribution and morphology in the intestinal wall. Calretinin, a calcium-binding protein expressed in intrinsic nerve fibers and ganglion cells, is generally absent in aganglionic colonic segments, serving as a reliable negative marker for Hirschsprung's disease. The combination of these two markers can improve diagnostic accuracy by clarifying equivocal findings on HE staining and aiding in the identification of the transitional zone.

The interpretation of IHC results requires specialized expertise in anatomic pathology, as staining patterns can vary depending on the quality of fixation, tissue handling, and immunostaining techniques. Therefore, IHC findings should not be interpreted in isolation but rather integrated with conventional histopathological evaluations. This combined approach may improve diagnostic reliability, reduce the risk of false-negative or false-positive interpretations, and facilitate accurate planning of segmental resection surgery.

Conclusion

In our study, calretinin demonstrated a slightly higher diagnostic value than S100 in detecting Hirschsprung's disease, with better sensitivity and

specificity. Although calretinin showed slightly higher sensitivity, no statistically significant difference was observed between the two markers. Therefore, these findings should be interpreted cautiously and require confirmation in larger, multicenter studies.

Conflict Interest

The authors declare no conflicts of interest

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