East African Medical Journal Vol. 102 No. 12 December 2025
DIAGNOSTIC UTILITY OF CALRETININ IN SUSPECTED CASES OF HIRSCHSPRUNG'S DISEASE FROM COLORECTAL BIOPSIES TAKEN FROM CHILDREN AT THE KENYATTA NATIONAL HOSPITAL IN NAIROBI, KENYA- A RETROSPECTIVE CASE SERIES STUDY

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ABSTRACT

***Background*: Hirschsprung's disease (HSD) is a pediatric colonic disorder marked by the absence of ganglion cells in the mucosa and myenteric plexi, causing functional intestinal obstruction. Diagnosis relies on rectal biopsy with Hematoxylin and Eosin (H&E), but subjectivity in ganglion cell recognition limits accuracy. Ancillary stains such as acetylcholinesterase have drawbacks, making Calretinin, which is a calcium-binding protein with immunoreactivity in ganglion cells and nerve fibers, the preferred marker. Its absence in the submucosa and myenteric plexus confirms HSD.**

***Objective*: To determine the diagnostic utility of Calretinin in suspected HSD cases from colorectal biopsies at Kenyatta National Hospital.**

***Methodology*: Paraffin blocks archived between January 2018 and December 2022 were retrieved. Slides were stained with H&E for initial diagnosis, then subjected to Calretinin immunohistochemistry (IHC) to assess ganglia and nerve fiber expression. Results were then analyzed using SPSS.**

***Results*: Forty-eight cases were studied; median age was 1 year (range: 3 days-9 years), with males comprising 56.3%. Five biopsies (10.4%) were inadequate. Of 43 evaluable cases, 23 showed ganglion cells, and 20 were negative on H&E. Calretinin stained ganglion cells in 22 cases and was absent in 21, including one missed by H&E. Hypertrophied nerve fibers were consistently present in ganglion-**

negative cases. Agreement between H&E and Calretinin was excellent (κ = 0.953, p<0.001).

Conclusion: **Calretinin IHC demonstrates high sensitivity, specificity, and diagnostic accuracy, with excellent agreement to H&E, supporting its role in confirming HSD.**

INTRODUCTION

Hirschsprung's disease (HSD) is a pediatric colonic disorder defined by the absence of ganglion cells in the mucosa and myenteric plexi. Its incidence is about 1 in 5,000 live births, making it a leading cause of functional intestinal obstruction in children[1,2]. Normal gastrointestinal function depends on the enteric nervous system (ENS), derived from neural crest cells forming the myenteric (Auerbach) and submucosal (Meissner) plexi. In HSD, ENS colonization fails, causing obstruction and proximal megacolon [3,4]. Classification of HSD depends on aganglionic segment length; short segment (up to sigmoid) and long segment (proximal to sigmoid), accounting for 80% and 20% of cases, respectively. Variants include total colonic, total intestinal, ultra-short, and suspended HSD [4]. Clinically, neonates present with failure to pass meconium within 48 hours, vomiting, distension, and enterocolitis. Later diagnoses manifest as chronic constipation, vomiting, and failure to thrive [5]. In Africa, delayed presentation worsens outcomes as shown by studies in Nigeria, Kenya, and Tanzania [6]. Children with HSD suffer reduced quality of life due to fecal incontinence, constipation, and caregiver anxiety, with severe socio-economic impact in developing countries, where it poses a diagnostic challenge in resource-limited settings [1].

Diagnostic evaluation includes radiology. Contrast Enema (CE) and Anorectal Manometry (ARM) are screening tools, followed by biopsy for confirmation [7]. Diagnosis is confirmed through positive rectal biopsies, with accuracy dependent on biopsy site, size, depth, type, and surgical skill [8]. Histological confirmation is traditionally achieved with Hematoxylin and Eosin (H&E) staining, but interpretation relies heavily on the pathologist's expertise. Senior pathologists identify ganglia and hypertrophic nerve fibers with relative ease, whereas junior pathologists face higher rates of false positives and negatives [9,10,11].

To overcome these challenges, ancillary stains were introduced. Acetylcholinesterase (AChE) histochemistry highlights neuronal activity but requires frozen section processing, limiting its utility in resource-constrained settings. Technical difficulties, interpretation challenges, and false positives further reduce its reliability [12,13] hence calretinin immunohistochemistry (IHC) has become the preferred ancillary test [9]. Calretinin, a calcium-binding protein expressed in submucosal ganglia and some myenteric ganglion cells, serves as a marker for intrinsic mucosal innervation. Complete absence of calretinin expression is highly specific and sensitive for HSD [7]. Unlike H&E, calretinin interpretation is binary (positive or negative), reducing reliance on pathologist experience [13]. Beltman et al. reported sensitivity and specificity of 96% and 100%, respectively, in experienced hands, and superior diagnostic accuracy compared with AChE and H&E among less-experienced pathologists [14]. Morris et al. demonstrated calretinin's superiority over AChE in cases without nerve hyperplasia or with superficial biopsies, noting its expression as early as 22 weeks of gestation, enabling earlier diagnosis [15]. In Senegal, Abdou

observed high concordance between H&E and calretinin. The sensitivity of calretinin was 93.75%, and the specificity 89.47%, with calretinin detecting some of the cases missed by H&E [16]. Despite its diagnostic advantages, accessibility of IHC remains limited in Africa due to high costs and a shortage of trained pathologists [17].

At the KNH, diagnosis of HSD is made using H&E staining alone. There has been no introduction of ancillary tests to aid in the diagnosis of HSD at the KNH, even though immunohistochemistry is currently being used worldwide as an invaluable diagnostic aid. This study will inform health policy on training and advocacy for sustainable, accessible diagnostic techniques and equitable resource distribution.

MATERIALS & METHODS

This was a retrospective case series study in which colonic/rectal biopsies of suspected cases of Hirschsprung disease were retrieved from January 2018 to December 2022 from the archives of Kenyatta National Hospital (KNH), which is the biggest referral hospital in East and Central Africa, located in Nairobi, Kenya. Cases were identified from the Human Pathology and Anatomic Pathology records department. The study population consisted of children under 12 years with a suspected diagnosis of HSD. A total of 244 cases were identified within the 5 years. Sample size was calculated using Buderer's formula. A stratified random sampling method was used to select 48 cases. Forty-eight (48) cases were selected according to the sample size calculation, and the blocks were retrieved and included in the study. Twenty-four (24) of the cases selected had been previously diagnosed as HSD on H&E, and the other 24 cases had been diagnosed as non- HSD. Colorectal blocks from suspected HSD cases were sectioned and stained with H&E per the KNH protocol, then examined microscopically for ganglion cells. Presence of ganglia indicated non-HSD, while absence with hypertrophied nerve fibers confirmed HSD. Blocks were further stained with Calretinin (Clone DAK-Calret 1); positive expression (excluding mast cells) denoted non-HSD, and negative expression indicated HSD. Two senior pathologists at UON, blinded to case details, independently interpreted both H&E and Calretinin slides, with findings systematically recorded by the primary investigator. A normal gut section from the appendix was used as a positive calretinin control in the study. Tissue blocks were handled as per the KNH histopathology laboratory SOP for routine IHC and H&E.

Reports meeting the inclusion criteria were retrieved by the principal investigator (PI) from the University of Nairobi Department of Human Anatomy records office, and each case was assigned a unique study number derived from the Kenyatta National Hospital report number. Clinical and demographic data were extracted from request forms and entered into a structured data collection tool, together with details of biopsy site and type. Corresponding paraffin-embedded specimens were recovered from the histopathology archives and stained with Hematoxylin and Eosin (H&E) and Calretinin immunohistochemistry (DAKO, Clone DAK-Calret 1) according to standard protocols. Biopsy adequacy, defined by the microscopic presence of submucosa, was documented. All slides were subsequently reviewed by the PI and two consultant pathologists, and the presence or absence of ganglion cells and hypertrophic nerve fibers was systematically recorded.

Data was collected on the data collection tool and entered into Microsoft Excel. Histologic data were presented using pictograms. Data was transferred to SPSS version 21.0 for

statistical analysis. Data analysis was done using Cohen's kappa coefficient (with a statistically significant level of $P < 0.05$). This was used to evaluate the level of agreement between the Calretinin IHC and H&E (gold standard). The diagnostic values, such as sensitivity and specificity, PPV, and NPV of Calretinin IHC, were calculated.

The study received ethical approval from the University of Nairobi Department of Human Pathology and the KNH-UON Ethics and Research Committee (Approval No. P911/12/2023). Permission to access tissue samples was granted by KNH and the UON Histopathology records department. For one altered diagnosis, the KNH Histology Laboratory addendum procedure was followed: the primary pathologist was notified, the clinician informed, the patient contacted, and the report archived in the records. To ensure confidentiality, no personal identifiers were used; unique study numbers linked to laboratory records were applied throughout.

RESULTS

In the period of review, 48 out of 244 histological biopsies, with a clinical suspicion of HSD, were selected using random stratification. Twenty-four (24) of the 48 cases had been initially histologically confirmed as HSD using H&E alone, while the remaining 24 had been diagnosed as non-HSD after H&E evaluation. That is, 24 cases had no ganglion cells (HSD), and in the other 24 cases, ganglion cells were identified (NHSD). All 48 cases were then stained with the gold standard H&E and then with Calretinin IHC (Clone DAK-Calret 1). The results are presented.

Demographic characteristics of the patients

The characteristics of the patients show that the median age was 1.0 year (IQR, 1.8 months – 3.5 years), where the minimum and maximum ages were 3.0 days and 9.0 years, respectively. The majority of the patients were male (56.3%).

Table 1

Characteristics of the patients

Characteristic	Frequency	Percent (%)
Age in Years		
≤1	28	58.3
2 – 5	15	31.3
6 – 9	5	10.4
Sex		
Male	27	56.3
Female	21	43.8

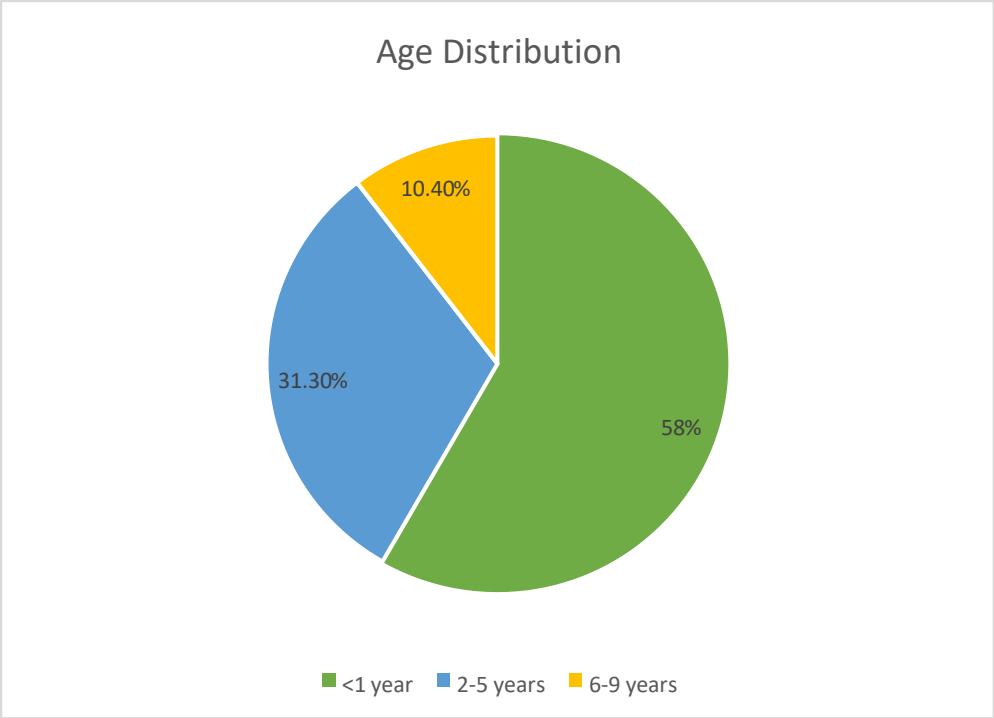


Figure 1: Pie Chart demonstrating the age distribution of the patients.

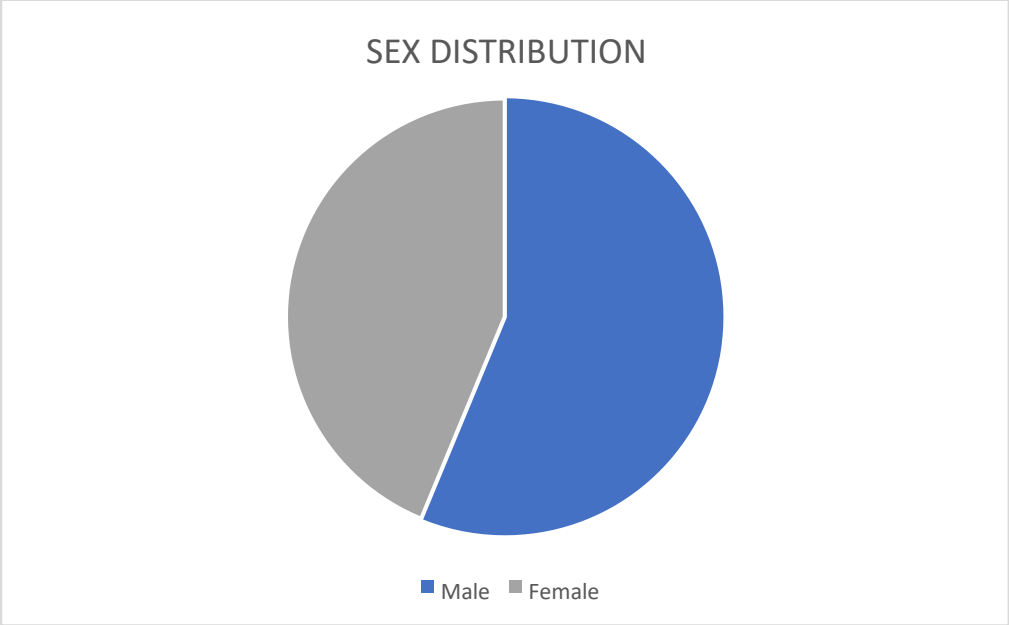


Figure 2: Pie chart demonstrating the sex distribution of the patients.

Table 2

Site of biopsy

	Frequency	Percent (%)
Colon	11	22.9
Colon/Rectum	12	25.0
Rectum	25	52.1

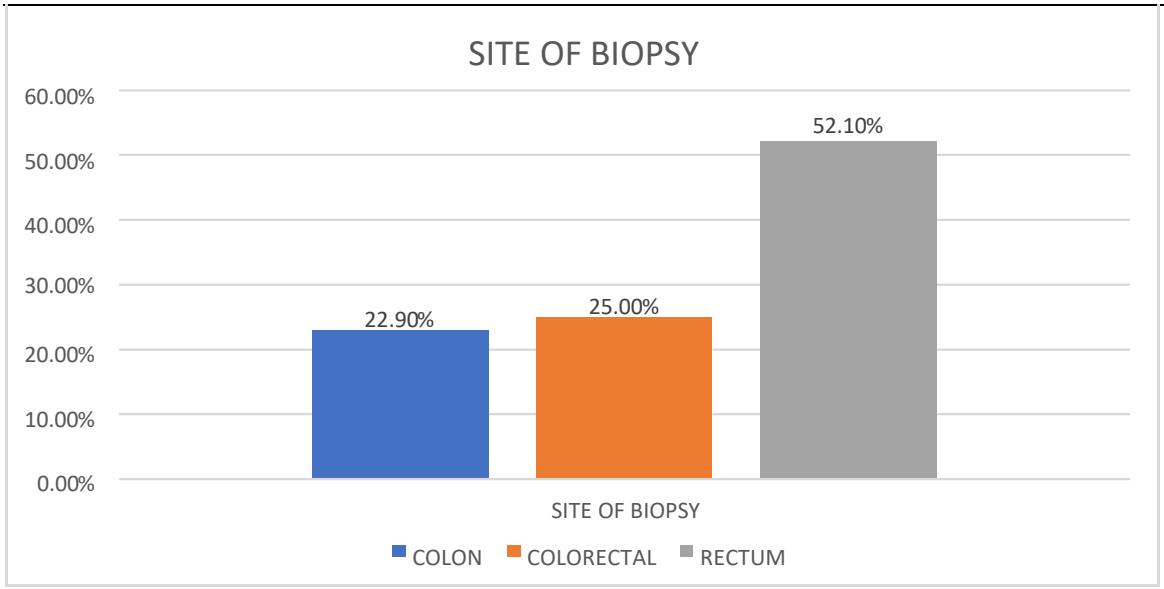


Figure 3: Bar chart representing the various sites of biopsy

Table 3

Type of biopsy

Type	Frequency	Percent (%)
1. Diagnostic Rectal Biopsy	26	54.2
2. Levelling Biopsy	9	18.8
3. Distal wall resection/pull-through segment	13	27.1

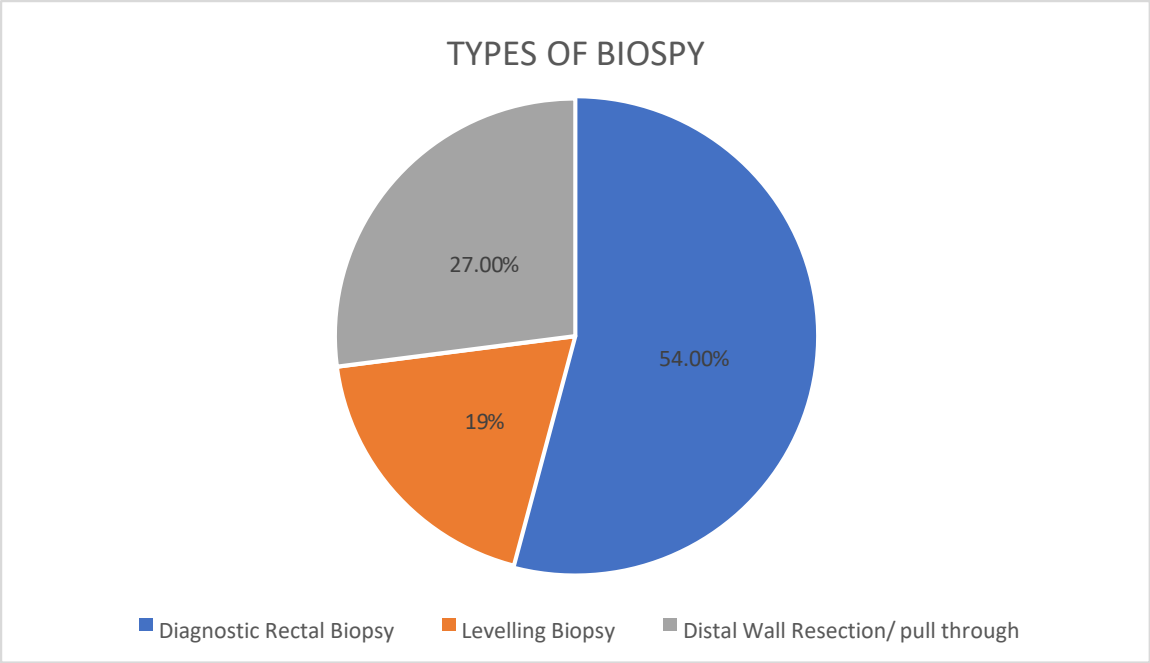


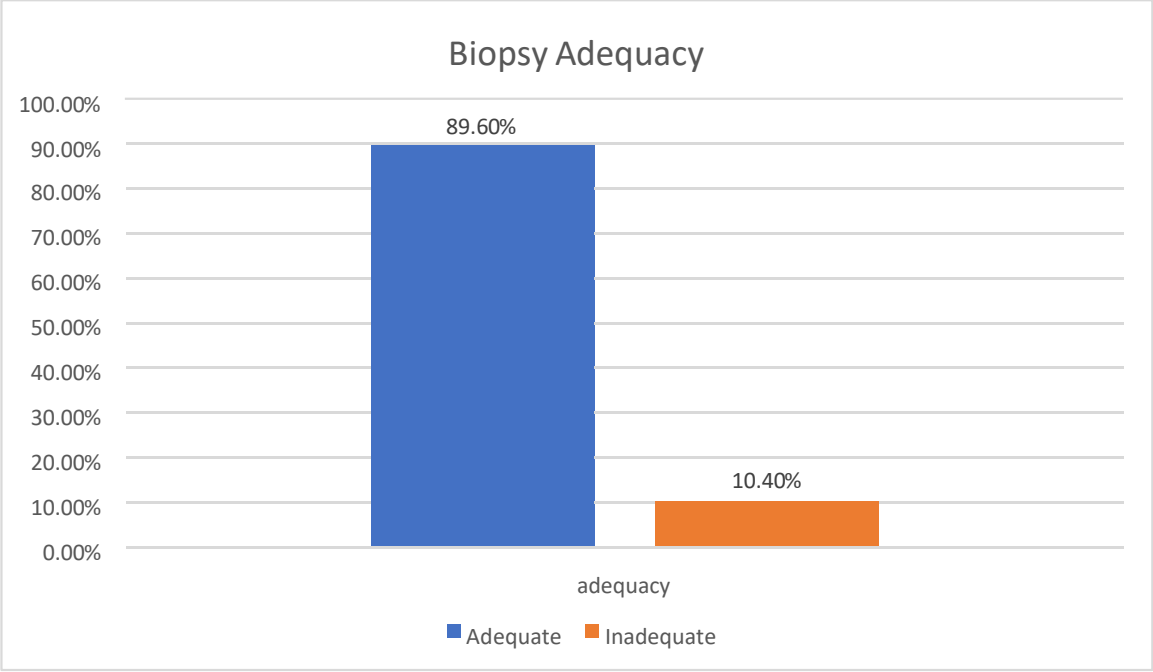
Figure 4: Pie chart demonstrating the various types of biopsies that were taken.

Table 4

Adequacy of specimens received

Type	Frequency	Percent (%)
Adequate	43	89.6
Inadequate	5	10.4

Figure 5: Bar chart demonstrating the adequacy of the biopsies received.



Clinical presentation

Thirteen (13) out of the 48 reports retrieved had clinical presentations recorded. Table 5 shows the frequency of each of the symptoms recorded.

Table 5

Clinical presentation

Clinical Signs and Symptoms	Frequency	Percent (%)
Chronic constipation	8	62
Delayed passage of meconium	2	15
Abdominal distension	2	15
Intestinal obstruction	1	8

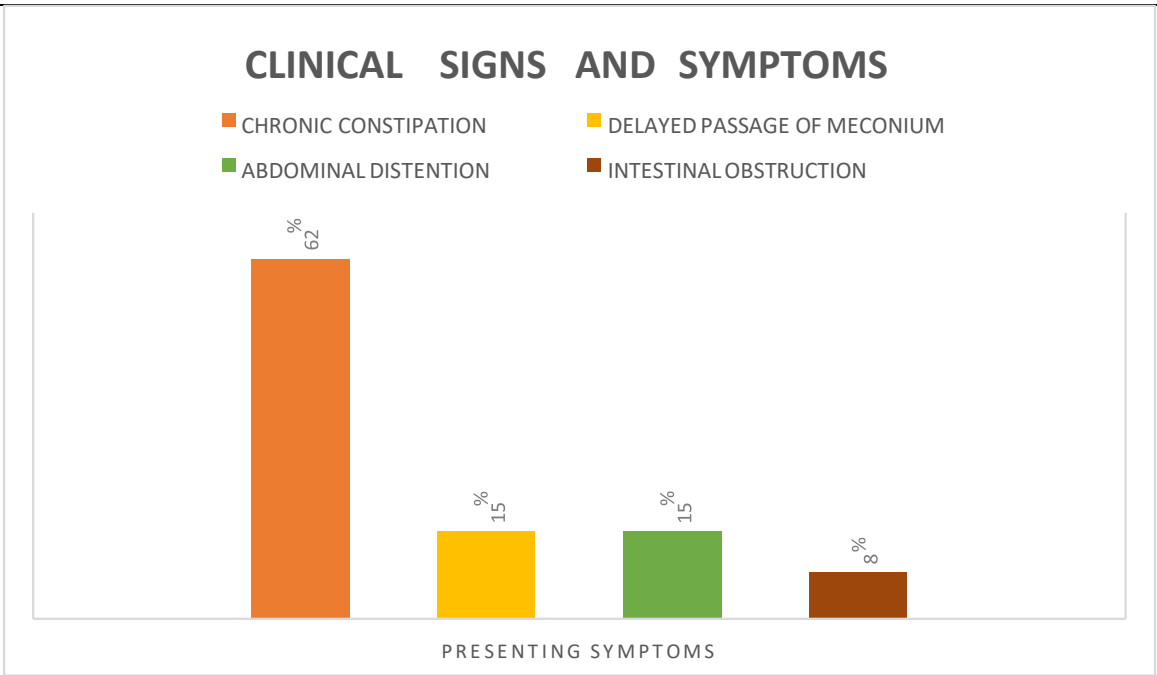


Figure 6: Bar Chart demonstrating the frequency of the clinical signs and symptoms experienced by the patients.

Hypertrophied Nerve Fibers (HNF)

Table 6 represents the presence of HNF seen

HNF	Frequency	Percent (%)
Present	21	49
Absent	22	51

Agreement between the gold standard H&E and Calretinin IHC

Cohen's kappa showed that there was excellent agreement between H&E and Calretinin IHC, $\kappa = 0.953$ ($p<0.001$). Concordance was at 42/43 = 97.7% (i.e., True Positive=22, and True Negative = 20).

Table 7
Pathologists H&E and IHC inter-rater agreement

	Kappa	p-value
Overall agreement	0.953	<0.001

Diagnostic utility of Calretinin

The sensitivity of calretinin was 95.7% (95% CI, 78.1% - 99.9%), the specificity of 100.0% (95% CI, 83.2% - 100.0%), and the overall accuracy of 97.7% (95% CI, 87.7% - 99.9%). The positive predictive value (PPV) was 100.00% (95% CI, 84.6% - 100.0%), and the negative predictive value (NPV) was 95.2% (95% CI, 74.6% - 99.3%).

Table 8
Diagnostic utility of Calretinin

		H & E				
	Ganglion Cells	Positive	Negative	PPV	NPV	
Calretinin	Positive	22	0	100.0%		
	Negative	1	20		95.2%	
	Sensitivity	95.7%				97.7%
	Specificity		100.0%			
	Accuracy					

HISTOPATHOLOGY PHOTOMICROGRAPHS
HAEMATOXYLIN AND EOSIN STAINS

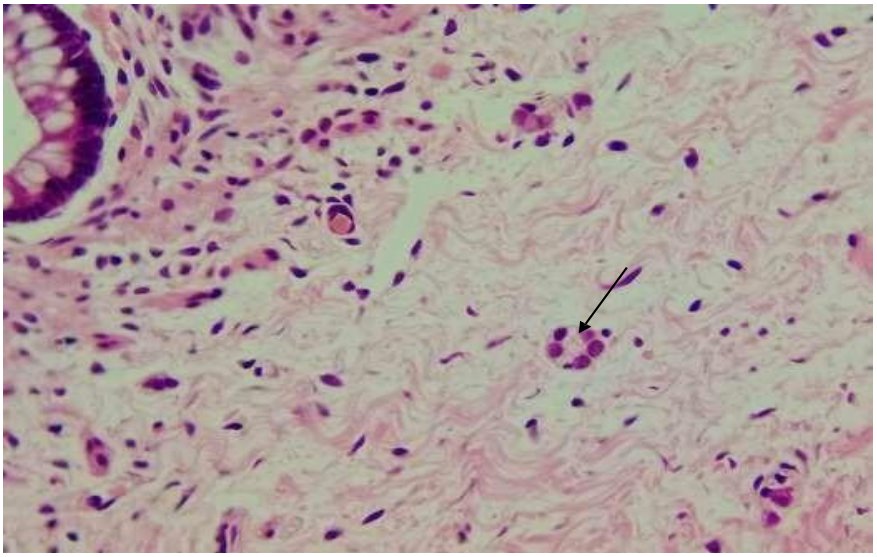


PLATE 1: NON-HIRSCHSPRUNG DISEASE
Mature ganglion cells (black arrow) at original magnification (20×)

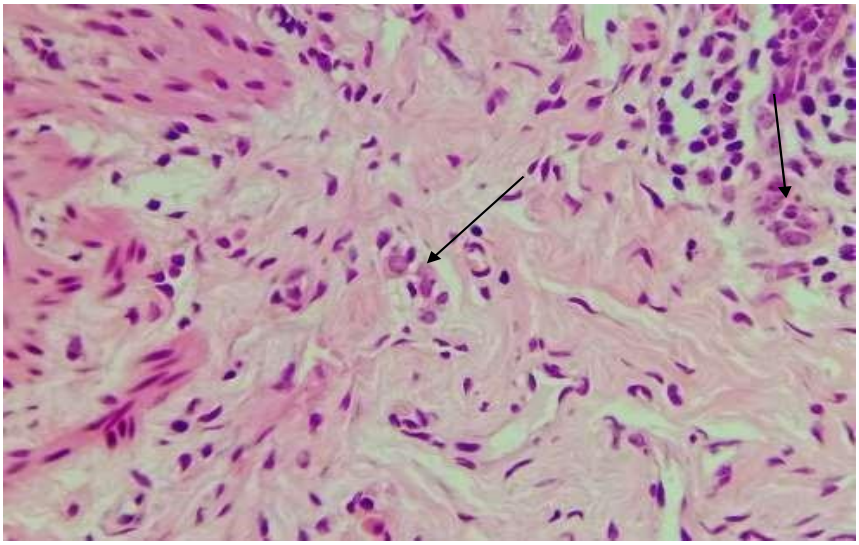


PLATE 2: H&E NON-HIRSCHSPRUNG DISEASE
Mature ganglion cells (black arrows) at original magnification (20×)

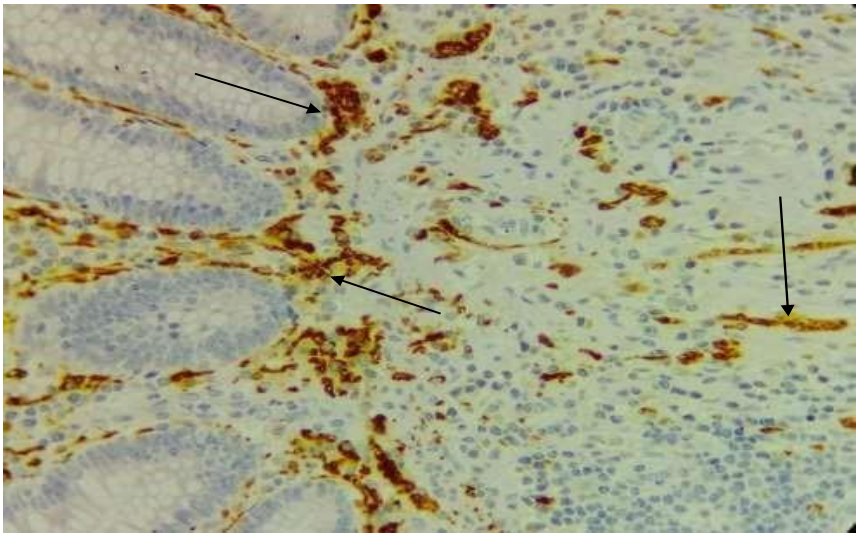


PLATE 3: POSITIVE CONTROL
Calretinin-positive ganglion cells and nerve fibres (black arrows) in a non-Hirschsprung disease biopsy. Original magnification 10×.

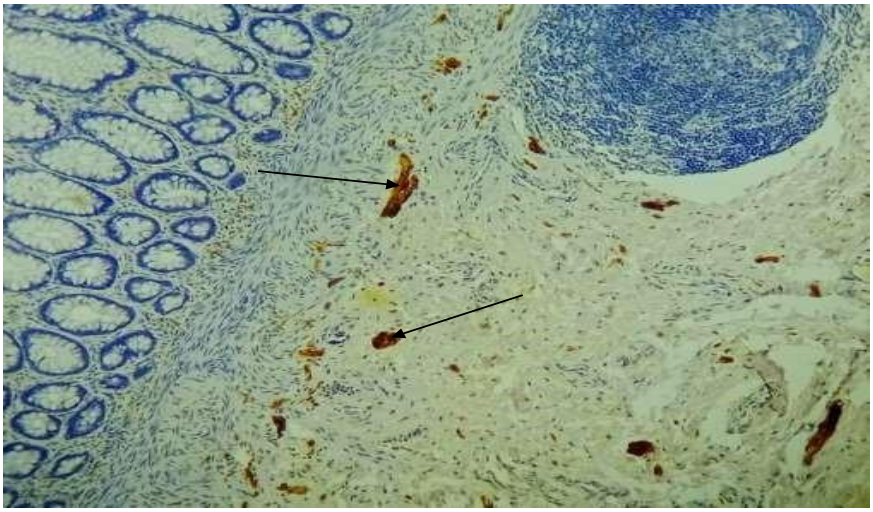


PLATE 4: IN NON-HIRSCHSPRUNG DISEASE

Calretinin-positive ganglion cells and nerve fibres (black arrows) in a non-Hirschsprung disease biopsy. Original magnification 10×.

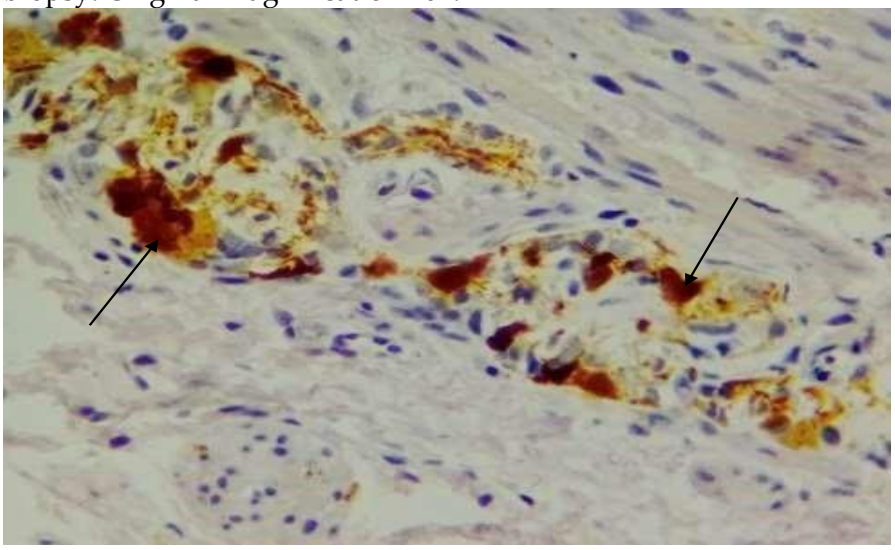


PLATE 5: IN NON-HIRSCHSPRUNG DISEASE

Calretinin-positive ganglion cells and nerve fibres (black arrows) in a non-Hirschsprung disease biopsy. Original magnification 40×.

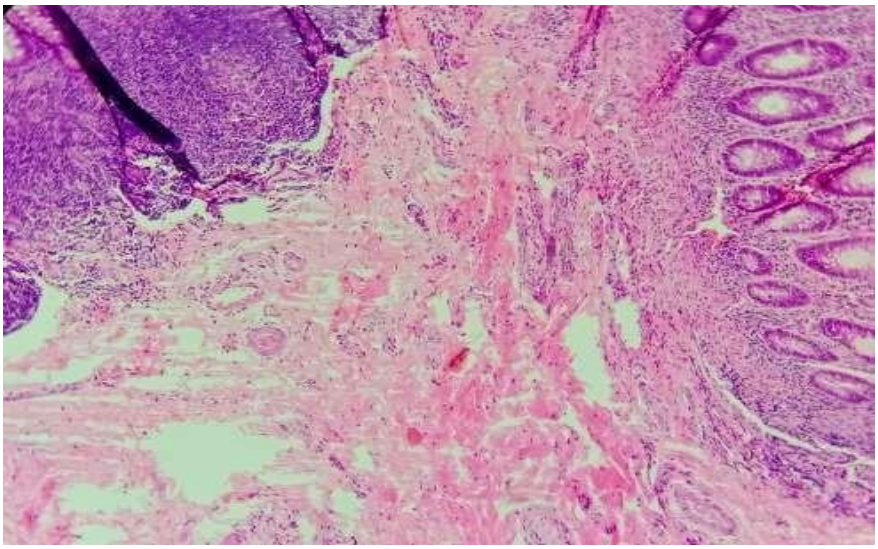


PLATE 6: H&E HIRSCHSPRUNG DISEASE
H&E staining is performed on a patient with Hirschsprung disease. No ganglion cells seen. Original magnification 10×.

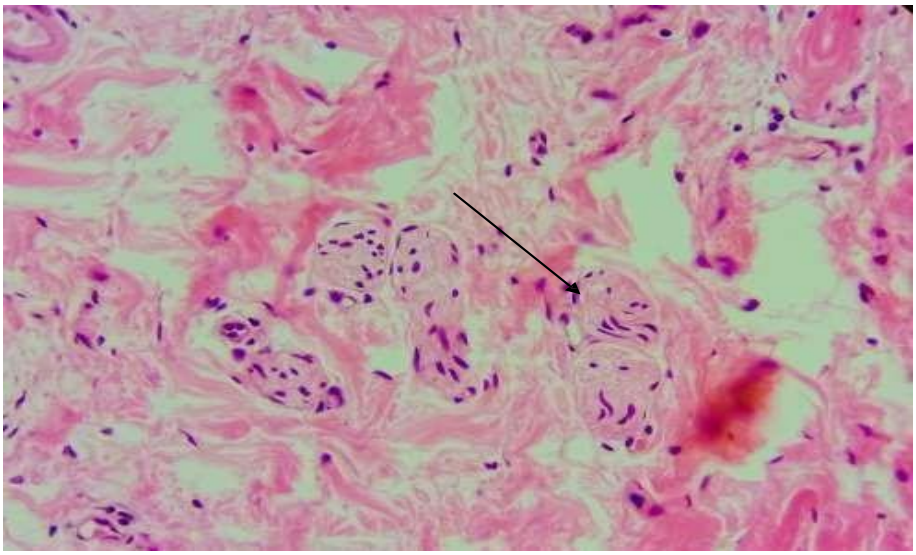


PLATE 7: H&E HIRSCHSPRUNG DISEASE (HYPERTROPHIED NERVE FIBRES)
H&E staining is performed on a patient with Hirschsprung disease. Hypertrophied nerve fibres (black arrows) with no ganglion cells seen. Original magnification 20×.

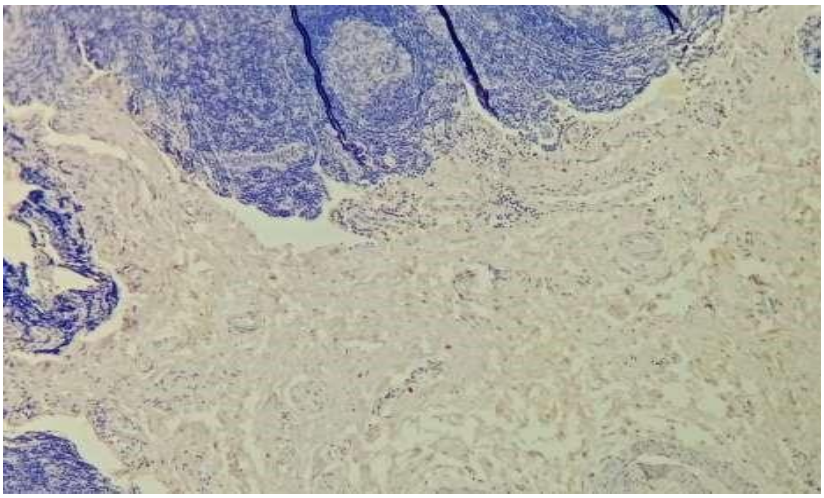


PLATE 8: CALRETININ IHC – HIRSCHSPRUNG DISEASE
Absent Calretinin staining in a patient with HSD at original magnification 10×.

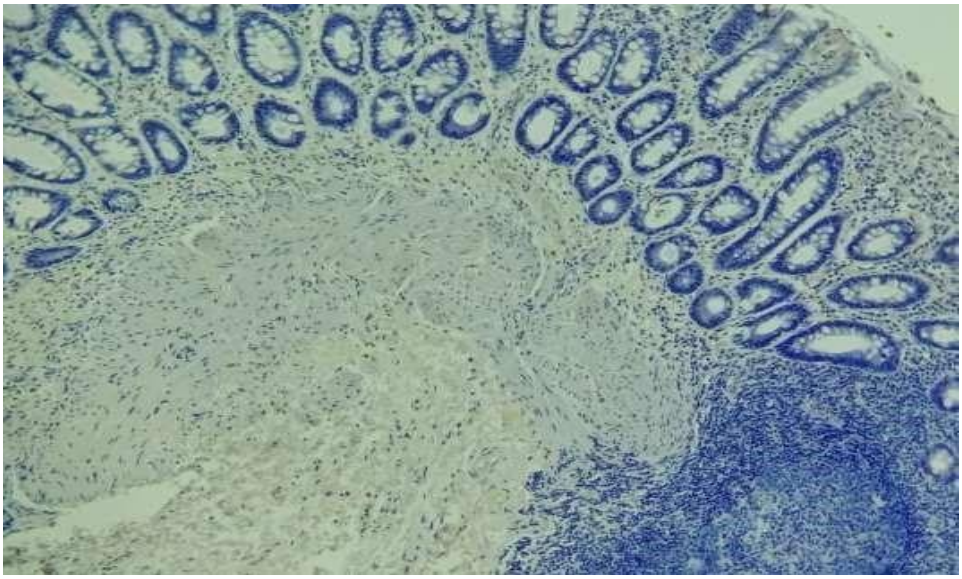


PLATE 9: CALRETININ IHC - HIRSCHSPRUNG DISEASE
Absent Calretinin staining in a patient with HSD at original magnification 10×.

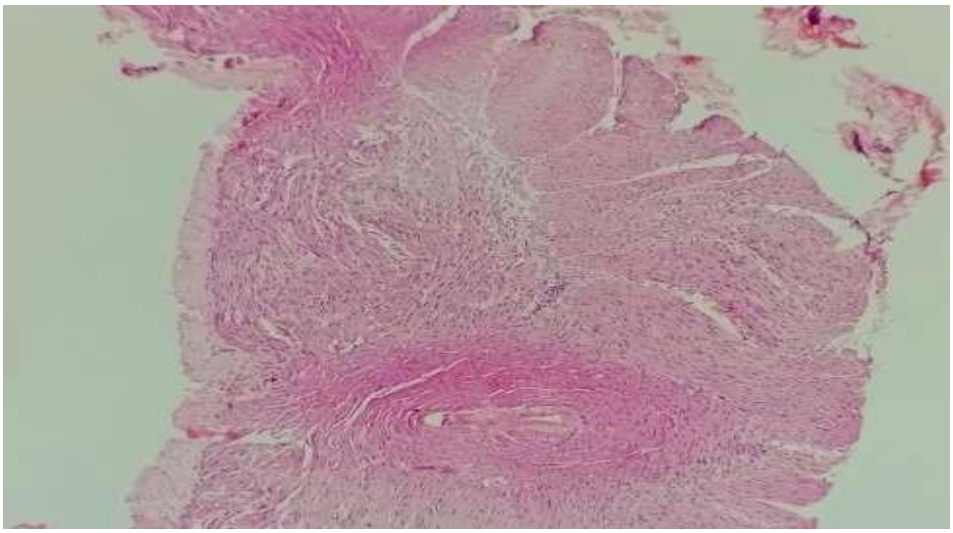


PLATE 10. INADEQUATE BIOPSY

The photomicrograph shows an inadequate rectal biopsy (without mucosa or submucosa) on H&E at original 10× magnification.

DISCUSSION

The diagnosis of HSD is made through the evaluation of ganglion cells in colorectal biopsy sections stained by the current gold standard H&E. This modality alone has proven to have challenges, as its interpretation is limited to the observer's ability to identify ganglia. Introduction of ancillary tests such as AChE and Calretinin IHC has been routinely utilized as an aid in the diagnosis of HSD [14,18]. Calretinin IHC is a marker for intrinsic mucosal innervation and was introduced in preference to AChE as it does not need special tissue handling, such as fresh frozen sections. Calretinin expression is considered a specific and highly sensitive finding in the diagnosis of HSD [7].

In this study, a total of 48 cases were included. Out of these, the median age of the patients was 1.0 year (IQR, 1.8 months – 3.5 years), while the minimum and maximum ages were 3.0 days and 9.0 years, respectively, findings comparable with those of Ongeti S in Kenya [19]. The majority of the patients were male (56.3%), while females represented were (43.7%) comparable to those found by Moore SW in South Africa [20]. The most common clinical presentations recorded were chronic constipation (62%), delayed passage of meconium (15%), abdominal distension (15%), and intestinal obstruction (8%), respectively. The clinical features study findings are similar to those observed in Tanzania, where the most common presentation was constipation [1].

All biopsies were incisional, with the commonest site of biopsy being the rectum at 52.1% (25/48), followed by colorectal at 25% (12/48) and colon only at 22.9% (11/48). In this study, the majority of the type of biopsies received were diagnostic rectal biopsies (54.2%), followed by resection/pull-through biopsies 27.1% and leveling seromuscular /full thickness biopsies. 5 out of the 48 (10.4%) of the biopsies were inadequate and hence inconclusive. 4 out of 5 (80%) of the inadequate biopsies were levelling biopsies, and 1 (25%) was a diagnostic rectal biopsy. These findings were slightly higher than those published by Vervloet looking at rectal biopsies in the diagnosis of HSD, found that 3% of the open

biopsies were inconclusive, and inconclusive results were due to insufficient submucosa [21].

Calretinin IHC offered no additional value in inadequate biopsies, similar to other published studies that recommended that re-evaluation (deeper) and rebiopsies be done to rule out HSD [22,23]. Of the 43 cases, 23 were positive for ganglion cells, and 20 were negative for ganglion cells on H&E. Calretinin positively stained ganglion cells in 22 of the cases; it was not expressed (negative) in 21 of the cases, including one that had been missed by the routine H&E. Hypertrophied nerve fibers were seen in all cases that were negative for ganglion cells. Cohen's kappa showed that there was excellent agreement between H&E and Calretinin IHC, $\kappa = 0.953$ ($p<0.001$). Concordance was at 42/43 = 97.7% (i.e., True Positive=22, and True Negative = 20, and 1 False positive.

In the diagnostic utility of Calretinin, its sensitivity was 95.7% (95% CI, 78.1% -99.9%), specificity of 100.0% (95% CI, 83.2% - 100.0%), and overall accuracy of 97.7% (95% CI, 87.7% - 99.9%). The positive predictive value (PPV) was 100.00% (95% CI, 84.6% - 100.0%), and the negative predictive value (NPV) was 95.2% (95% CI, 74.6% - 99.3%). These findings align with those of Rakshani et al, who demonstrated that Calretinin IHC offered significantly higher specificity and accuracy than H&E (P<0.0001) [11]. Similarly, Hiradfar et al. reported Calretinin sensitivity of 93.3% and specificity of 100% in full-thickness biopsies with predictive values of 100% and 93.8%, confirming its reliability in SRBs [24]. Mukhopadhyay et al. in India found Calretinin IHC to have a sensitivity of 100% and 97.44% specificity [25]. In Senegal, Abdou observed high concordance between H&E and calretinin. The sensitivity of calretinin was 93.75%, and the specificity 89.47%, with calretinin detecting some of the cases missed by H&E.

CONCLUSION

The gold standard H&E is still recommended in our setup due to its high sensitivity and specificity amongst our pathologists. Calretinin IHC is a good ancillary test to be utilized in the diagnosis of HSD. Calretinin IHC has a high sensitivity and specificity as compared to the standard H&E in the identification of ganglion cells. The diagnostic accuracy was 97.7%, and there was excellent agreement, $\kappa = 0.953$ ($p<0.001$), between IHC and the standard H&E. Clinical Pathological correlation between pathologists and pediatric surgeons is recommended in order to reduce the number of inadequate rectal biopsies (absence of submucosa).

Conflict of Interest

Authors declare no conflict of interest.

CRediT authorship contribution Statement

*Dr. Melaki Boy Juma: Principal Investigator and writer of the original draft, Dr. Wairimu Waweru: Supervisor and project administrator, Dr. Zuriel Daniel: Supervisor and project administrator, Dr. Peter Maturi Mwamba: supervisor and project administrator, Dr. Steph Apondi: Writing- review and editing, Dr. Nyakio Mburu: Writing- review and editing, Dr. Tim Jumbi: Supervisor and project administrator

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