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**Original Article**

# Immunohistochemical Diagnostic value of Calretinin in Hirschprung Disease

**Zainab Azis[1*], M. Husni Cangara[1], Andi Alfian Zainuddin[2], Syarifuddin Wahid[1], Nita Mariana[3], Upik A. Miskad[1].**

*[1]Department of Anatomical Pathology, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia;*
*[2]Department of Public Health and Community, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia*
*[3]Division of Pediatric Surgery, Department of Surgery, Faculty of Medicine, Universitas Hasanuddin, Makassar, Indonesia*

*Corresponding author: Zainab Azis
zainab8azis@gmail.com

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**ABSTRACT**

***Introduction****: Hirschsprung's disease (HD) is characterized by the absence of ganglion cells in Meissner's plexus and Auerbach's plexus which used to be assessed by histopathological examination using Hematoxylin and Eosin (H&E). In some cases is difficult to assess ganglion cells, thus requiring additional examination in the form of calretinin immunohistochemistry to detect the presence of ganglion.* ***Methods****: The 59 samples of suspected HD were analyzed by H&E staining followed by calretinin immunohistochemical staining. This study aimed to assess the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of calretinin in HD diagnostics.* ***Results:*** *The immunohistochemistry of calretinin showed a good diagnostic value in detecting aganglionosis in patients with suspected HD with a sensitivity value = 97.7%; Specificity = 100%; Positive Presumptive Value (PPV) =*

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*100%; Negative Presumptive Value (NPV) = 93.7%; Accuracy= 98.3%. Thus, calretinin immunohistochemistry can be relied upon as an additional method in the diagnosis of HD*



## 1. INTRODUCTION

Hirschsprung's disease (HD) is a congenital disease caused by failure of neural crest migration in the developmental phase of the intestine at 5 to 12 weeks of gestation [1,2]. There are two plexuses in the nervous system in the intestine are submucosal (Meissner's plexus) and myenteric (Auerbach's plexus) which function in motility (contraction and relaxation), absorption, secretion, and blood flow [3,4]. Clinical symptoms of patients with HD in infants and children can appear symptoms of constipation, abdominal distension, nausea, and vomiting. Newborns are characterized by not passing meconium since 24 hours of birth [5].

HD is the most common disease in neonates. In Indonesia, the prevalence of congenital abnormalities reaches 1 per 3,250 live births [6]. The incidence of HD is higher in men than women at around 4:1 [7].

The Gold standard staining for diagnosing HD is *Hematoxylin and Eosin* (H&E) in many countries because apart from being economical it is also a routine stain. However, there are cases of difficult aganglionic diagnosis with H&E staining. In neonates, ganglion cells are difficult to identify because of their small size, and the biopsy is difficult to diagnose as HD or non-HD [8,9]. Therefore, the additional immunohistochemical examination is needed to increase accuracy in HD diagnosis. In some studies, Calretinin is useful in detecting ganglion cells. In ganglionic tissue, calretinin is expressed in the nucleus and cytoplasm of ganglion cells in the Aurbach plexus and Meisner plexus, while in aganglionic tissue calretinin is not expressed in both the Aurbach and Meisner plexuses [10,11]. Calretinin immunohistochemistry has advantages in the diagnosis of Hirschsprung's disease: simple interpretation and easy application, use of formalin blocks [12].

## 2. METHODS

This study is a retrospective study based on medical record data and histopathology. We collected fifty-nine samples clinically diagnosed as suspected HD and classified them d into aganglionic and ganglionic. Samples were taken from a biopsy or resection of colorectal tissue in suspected HD aged <18 years. Colonic or rectal biopsy blocks that did not reach the submucosal layer were excluded from this study. Immunohistochemical examination of Calretinin using a paraffin block was cut with a thickness of 4 µm then stained with primary antibody calbindin 2 (29kDa calbindin) rabbit polyclonal antibody with a dilution of 1;100. then incubated with Envision-labeled polymer (Dako) for 60 minutes. Immunohistochemistry of calretinin was positive if it was stained

brown diffusely in the cytoplasm or nucleus of ganglion cells in the Aurbach and Meisner plexus. The data was processed by descriptive statistical techniques and also processed by statistical analysis techniques carried out with SPSS 20 software for windows. Descriptive analysis to describe the characteristics of the basic data in the form of frequency distribution on age, gender, type of sample. Then, the sensitivity, specificity, positive predictive value, negative predictive value, and immunohistochemical accuracy of calretinin were carried out.

For diagnostic value tests in determining:

Sensitivity = True positive / (True positive + False negative) x100%

Specificity = True negative / (True negative + False positive) x100%

PPV = True Positive / (True Positive + False Positive) x100%

NPV = True Negative / (True Negative + False Negative) x100%

Accuracy= True positive + true negative/ (true positive + false positive + false negative + true negative) x 100%

## 3. RESULTS

The total sample of the study was 59 cases. The total majority of the sample population is male as many as 35 samples (59.3%). With the most common age 1 month – 1 year as many as 24 samples (40.7%). The most common type of surgery performed was biopsy, as many as 33 cases (55.9%). Using H&E staining, 15 cases (48%) were positive for ganglion cells from 59 cases. In positive immunoreactive calretinin staining on ganglion cells, 16 samples (27,1%) were found (table 1)

**Table 1. Demographic character overview of the study population**

| Sample Characteristics | n | % |
|---|---|---|
| **Sex** | | |
| Males | 29 | 58 |
| Females | 21 | 49 |
| **Ages** | | |
| <1 months | 7 | 14 |
| 1 6 months– 1 year | 23 | 46 |
| >1 year | 20 | 41 |
| **Sampels** | | |
| Biopsy | 24 | 48 |
| Resection | 26 | 52 |
| **H&E** | | |

| | | |
|---|---|---|
| Ganglionic | **8** | 16 |
| Aganglionic | 42 | 84 |
| **Calretinin** | | |
| Positive | **9** | 18 |
| Negative | 41 | 82 |

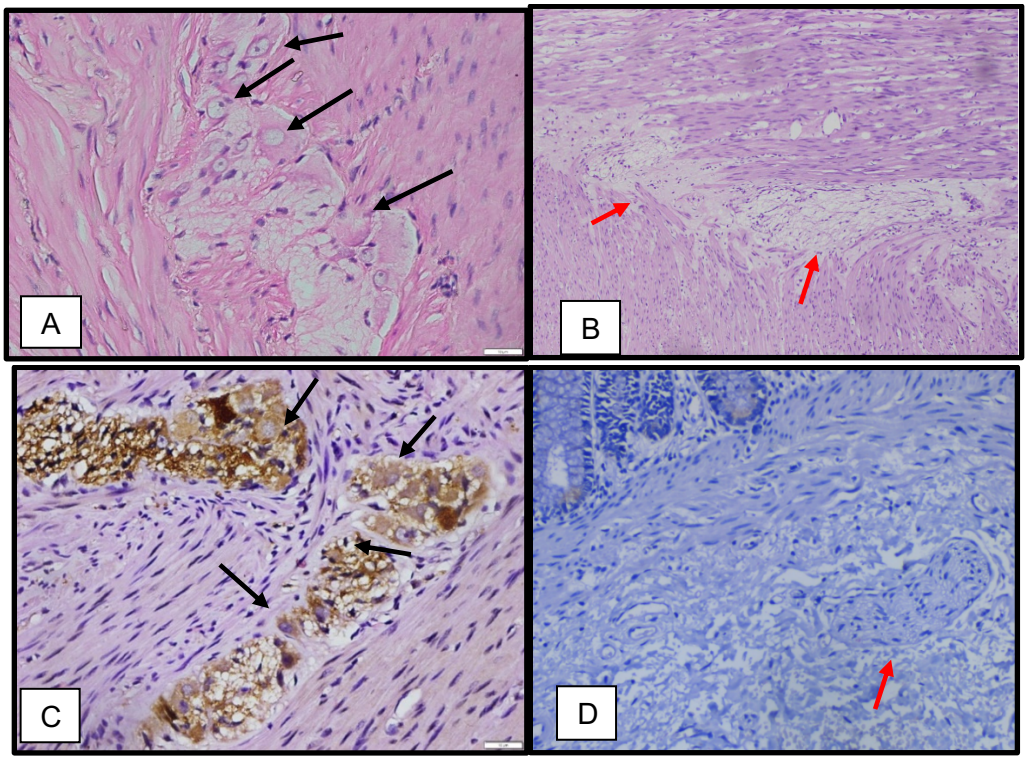


Figure 1. Ganglion cells in the external muscle. B, aganglionic nerve fibers. H&E staining C. Positive calretinin on ganglion cells D. Negative calretinin in aganglionic tissue (400x magnification, black arrows: ganglion cells, red arrows: aganglionic fibers)

**Table 2 Diagnostic test with H&E and calretinin immunohistochemical staining.**

| | H&E | | | | Total | (%) |
|---|---|---|---|---|---|---|
| | Negative (n) | % | Positive (n) | % | | |
| **Calretinin** | | | | | | |
| Negative | 43 | 100 | 0 | 0 | 43 | 100 |
| Positive | 1 | 6,3 | 15 | 93,8 | 16 | 100 |

| | | | | |
|---|---|---|---|---|
| Total | 44 | 15 | 59 | 100 |

The results of the diagnostic test, the sensitivity values obtained are: 97.7%; specificity: 100%; positive predictive value: 100%; negative predictive value: 93.7%; accuracy: 98.3%.

## 4. DISCUSSIONS

This study found that the male gender is slightly more than the female sex. These results are from previous Hirschsprung research conducted by Puri where the incidence of HD was slightly higher than women.[3] In research, in general, the incidence of HD is greater in men [13]. In this study, the highest incidence of HD was 1 month-montmonthsnerally, the diagnosis of HD is mostly established at the age of 0-30 days [14,15].

H&E staining is still widely used in the diagnosis of HD in developing countries because it is routine staining, low cost, and easy to use. According to Swenson and Agrawal, the use of H&E is not accurate in the diagnosis of HD in small biopsy tissue [8]. Lack of color contrast between ganglion cells and surrounding tissue of H&E staining in diagnosing Hirschsprung's disease requires an additional marker.

Calretinin is a calcium-dependent vitamin D binding protein that functions as a buffer against excess cytosolic calcium ions; calcium transport [16]. Calretinin is positively expressed in ganglion cells in the form of color in the nucleus and cytoplasm [17,18]. In the aganglionic segment, calretinin will not be expressed in the network of nerve fibers [19–21]. In this study, false negatives were obtained, this is due to the small size of the ganglion cells in the submucosal layer so with H&E staining it is difficult to see, but with calretinin staining the ganglion cells are very contrasting so they are easy to see. The presence of false positives and false negatives in the diagnosis of HD was reported in the Rhaksani study, 2016 which found false positives and false negatives in neonates [12].

In this study, the immunohistochemistry of calretinin obtained sensitivity values of 97.7%; specificity: 100%; positive predictive value: 100%; negative predictive value: 93.7%; accuracy: 98.3%. This is by Mukhopadhyay's study of the high sensitivity and specificity of calretinin to ganglion positivity [22].

## 5. CONCLUSION

Calretinin immunohistochemistry potential can replace H&E staining as the gold standard in diagnosing Hirschsprung disease

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**Conflict of Interest Statement:**

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.