**Original Research Article**

**Comparative study between use of calretinin and synaptophysin immunostaining in diagnosis of Hirschsprung disease**

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**ABSTRACT**

**Background:** Hirschsprung disease (HD) is diagnosed by absence of ganglion cells in rectal biopsy. In some cases, standard methods fail to diagnose aganglionosis. The aim of this study is to assess the diagnostic value of immunohistochemistry (IHC) of calretinin and synaptophysin compared to standard methods.

**Methods:** This prospective cross section study was conducted in Menoufia University hospitals, Egypt spanning the period between October 2017 to December 2018. Rectal biopsies of the clinically suspected HD patients stained with calretinin and synaptophysin and their results compared to the standard hematoxylin and eosin (H&E) stained sections.

**Results:** A total of 30 patients aged from 3 days to 2 years with a male to female ratio 11:4 were examined for rectal biopsies. Sections of 9 cases were diagnosed HD. In inadequate specimens, sensitivity and specificity of calretinin and synaptophysin (100%, 80%) and (100%, 85.71%) respectively were superior to the sensitivity (40%) and specificity (14%) of H&E. However, in adequate specimens, results of H&E, calretinin and synaptophysin were the same.

**Conclusions:** Immunohistochemical expression of calretinin and synaptophysin were conclusive, diagnostic and superior to the results of H&E stained section in inadequate. However, in adequate specimens calretinin and synaptophysin were consistent and confirmatory to the results of H&E sections.

**Keywords:** Calretinin, Hirschsprung disease, Immunohistochemistry

**INTRODUCTION**

Hirschsprung Disease (HD) is a developmental disorder of the enteric nervous system with incidence of 1: 5000, it is characterized by absence of ganglion cells in the myenteric and submucosal plexuses and hypertrophied nerve terminal along a variable portion of the distal intestine.¹ The rectum is always affected and in 90% patients the disease extends up to rectosigmoid region.²

The most widely accepted etiopathogenic hypothesis is based on a defect of craniocaudal migration of neuroblasts originating from the neural crest.³ The clinical features of HD include: history of failure to pass meconium after 24 hours of age, constipation, abdominal distension, bilious vomiting and failure to thrive.⁴ Approximately 80 - 90% of patients are diagnosed during the newborn period, however some patients present during infancy, childhood or even in adults usually with a chief complaint of chronic constipation.⁵

The main diagnostic investigations are megacolon appearance in barium enema and demonstration of absence of ganglion cells and hypertrophied nerve terminals in rectal biopsy.⁶

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The aim of surgical management of HD is resection of aganglionic segment of colon and restoration of bowel continuity. Three most common operations for the definitive management of this condition are the Swenson, Duhamel and the Soave procedures, although each has been modified following its original description, the three procedures continue to be used as the standard operations for the definitive treatment of this disease.

Hirschsprung disease (HD) is classically diagnosed or excluded by rectal biopsy, a method that take a sample a 2 to 3 mm of rectal mucosa and underlying submucosa. Different approaches are customized to evaluate rectal biopsies, but the foundation for diagnosis or exclusion of HD in most practices is microscopic examination of hematoxylin and eosin (H&E)-stained sections. High accurate identification of submucosal ganglion cells excludes HD, whereas aganglionic adequate sample is considered diagnostic.

However, adequate sampling is a serious concern for which limited published standards exist.

One of the approaches is to evaluate multiple H&E stained levels from each paraffin-embedded biopsy. This technique is applied in most pediatric pathology laboratories. The reliability of this technique relies on the observer's ability to accurately identify a ganglion cell in H&E stained sections. Although no universal agreement regarding the number of histological sections needed to diagnose HD has been approved, previous works relied on the histopathological examination of 50 serial H&E stained sections.

However, in some cases, H&E stained sections fail to diagnose agangliosis. HD remains a challenging diagnosis, especially among general surgical pathologists who assess these cases occasionally and are insufficiently experienced. In neonates, submucosal ganglionic cells can’t be identified easily because they are classically small and undifferentiated.

A number of ancillary methods have been introduced in efforts to simplify the challenges posed by H&E-based evaluation of rectal biopsies. Several immunohistochemical markers such as S-100 protein, neuron-specific enolase (NSE), calretinin and synaptophysin have been tried.

Calretinin is a vitamin D-dependent calcium-binding protein involved in calcium signaling, which has an important role in the organization and functioning of the central nervous system. Synaptophysin, a 38-KD membrane protein specific for the synaptic vesicles in the central and peripheral nervous systems is the main constituent of AChE storage compartments, and an important neuromuscular junction marker.

Calretinin immunohistochemical stain accurately identify ganglions through staining nucleus, cytoplasm and some nerve fiber, and so it’s used as additional diagnostic tool for HD.

Immunostaining of synaptophysin after formalin fixation may be used to identify hyperplasia of nerve fibers and stains cytoplasm of ganglion cells in non-HD specimens. It could be useful to demonstrate abnormalities of enteric innervation in rectal biopsies performed for suspected Hirschsprung's disease in the absence of acetylcholinesterase staining on frozen sections.

The aim of this study is to assess the diagnostic accuracy of calretinin and synaptophysin IHC staining in diagnosis HD and compare their results to the golden standard H&E stained sections.

METHODS

A prospective cross section study was conducted on 30 cases with clinically suspected HD. The enrolled participants in the study were all patients aged from 3 days to 2 years, presented to Pediatric Surgery Unit in General Surgery Department, Menoufia University hospitals, Egypt, spanning the period between October 2017 to December 2018.

Participant selection

All neonates and infants presented to Menoufia University hospitals with delayed passage of meconium or chronic constipation dating back since birth were enrolled in the study.

Informed consent was obtained from each patient’s parent or his legal guardian. This study has been approved by the Ethics Committee of Menoufia University Hospital.

All participants were undergone erect chest abdomen X-ray and barium enema.

Rectal biopsy

Rectal biopsy taken under general anaesthesia 2cm above dentate line with thickness 2-3mm from the posterior aspect of rectum (2-3 biopsies taken per time), hemostasis achieved by electrocauterization and suturing the defect with previously placed traction absorbable suture.

All specimens fixed in 10% neutral buffered formalin were sent to Pathology Department, Faculty of Medicine, Menoufia University for routine processing and preparation of H&E staining slides for routine assessment, at least 50 sections are evaluated for the presence of ganglion cells.

Rectal biopsies are classified into adequate and inadequate. Adequate biopsy defined to be 2-3mm thickness and submucosa represent at least one third of its
thickness. Inadequate biopsy contains insufficient submucosa or lymphoid follicles.\(^9\)

Four um thick section was cut for each marker on a positive charged slide for immunohistochemistry staining using calretinin and synaptophysin antibodies.

Diagnostic criteria for HD in rectal mucosal biopsy by H&E stained slides are absent ganglion cells and presence of hypertrophic nerve bundles in submucosa. Diagnostic criteria for non-HD in rectal mucosal biopsy: At least one ganglion cell is identified in one or more tissue sections.\(^1\)

**Immunohistochemical staining procedure for calretinin and synaptophysin**

Immunohistochemical staining was performed using antibodies: ready-to-use calretinin from cell Dako (Monoclonal Mouse Anti-Human Calretinin Clone DAK-Calret 1; Dako, Denmark) and ready-to-use synaptophysin from cell Dako (Monoclonal Mouse Anti-Human Synaptophysin Clone DAK-SYNAP; Dako, Denmark). Positive control slides were mesothelioma and neuroblastoma for calretinin for synaptophysin respectively. Negative control slides were prepared by omitting the primary antibodies from the staining procedure.

**Statistical analysis**

Statistical calculations were carried out using Statistical Package for the Social Science (SPSS) version 20 software (Chicago, IL, USA). The values for accuracy, specificity, sensitivity, positive predictive value, and negative predictive value were estimated for calretinin and synaptophysin IHC when compared with conventional histopathology (H&E) as a golden diagnostic standard.

**RESULTS**

**Baseline demographic data**

Mean age of infants was 12.08 months with Standard deviation (10.67 months). Male were 22 (73%), while females were 8 (27%) (Table 1).

**Adequacy of rectal biopsies and H&E stained sections**

Assessment of H&E stained section for each patient showed 18/30 (60%) adequate specimens and 12/30 (40%) inadequate specimens.

Hirschsprung Disease (HD) was diagnosed in 4 out of 18 of adequate specimens by demonstration of absence of ganglion cells in both lamina propria and muscularis mucosa.

In the specimens of adequate rectal biopsy, calretinin and synaptophysin IHC results in the identification of ganglion cells and nerve fibers were consistent with the results of H&E stained section (Figure 1 and Table 2).

![Figure 1: H&E stained slides. (A): A case of non-HD rectal mucosa, (B): submucosa showed ganglion cells (circle), (C): a case of HD rectal mucosa and (D): submucosa showed hypertrophied nerve fiber (arrow) and no ganglion. (H&E x400 for A and B, and x200 for C and D).](image)

Table 1: Baseline demographic data.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
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<td>Age</td>
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<tr>
<td></td>
<td>Median 11.8 months</td>
</tr>
<tr>
<td></td>
<td>Range 3 days - 2 years</td>
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<tr>
<td>Gender</td>
<td>Male 22</td>
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<tr>
<td></td>
<td>Female 8</td>
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<td></td>
<td>M/F ratio 11:4</td>
</tr>
</tbody>
</table>

However, in case of inadequate rectal biopsy, H&E failed to identify ganglion cells in 6 biopsies out of 7, which confirmed to be ganglionic by calretinin IHC (Figure 2).

While synaptophysin showed higher accuracy than H&E in inadequate specimens and identified ganglion cells in 5 specimens out of 7 ganglionic specimens (Figure 3). Calretinin truly identified the all 7 ganglionic biopsies in
case of inadequate specimens. Hematoxylin and eosin were conclusive in 2 specimens out of 5 specimens to be aganglionic, while synaptophysin was more sensitive and diagnosed 4 out of 5 specimens to be HD. Calreteinin diagnosed the whole 5 cases of HD (Table 3).

![Figure 2: Calretinin IHC stained slides. (A): a case of non-HD showed positive nucleocytoplasmic calretinin immunohistochemistry (IHC) expression in ganglia (circle), (B): and few adjacent nerve fiber, (C): a case of HD showed negative calretinin IHC expression, (D): few nerve fibers (arrows) exhibited positive calretinin expression. (Calretinin IHC x400 for A, B, C and D).]

Table 2: Accuracy measurement between H&E, calretinin and synaptophysin in diagnosis of Hirschsprung’s disease in adequate rectal biopsies.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>H&amp;E</th>
<th>H&amp;E %</th>
<th>Calretinin</th>
<th>Calretinin %</th>
<th>Synaptophysin</th>
<th>Synaptophysin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>4/4</td>
<td>100%</td>
<td>4/4</td>
<td>100%</td>
<td>4/4</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>14/14</td>
<td>100%</td>
<td>14/14</td>
<td>100%</td>
<td>14/14</td>
<td>100%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>4/4</td>
<td>100%</td>
<td>4/4</td>
<td>100%</td>
<td>4/4</td>
<td>100%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>14/14</td>
<td>100%</td>
<td>14/14</td>
<td>100%</td>
<td>14/14</td>
<td>100%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>18/18</td>
<td>100%</td>
<td>18/18</td>
<td>100%</td>
<td>18/18</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 3: Accuracy measurement between H&E, calretinin and synaptophysin in diagnosis of Hirschsprung’s disease in inadequate rectal biopsies.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>H&amp;E</th>
<th>H&amp;E %</th>
<th>Calretinin</th>
<th>Calretinin %</th>
<th>Synaptophysin</th>
<th>Synaptophysin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>2/5</td>
<td>40%</td>
<td>5/5</td>
<td>100%</td>
<td>4/5</td>
<td>80%</td>
</tr>
<tr>
<td>Specificity</td>
<td>1/7</td>
<td>14%</td>
<td>7/7</td>
<td>100%</td>
<td>6/7</td>
<td>85.71%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>2/8</td>
<td>25%</td>
<td>5/5</td>
<td>100%</td>
<td>4/5</td>
<td>80%</td>
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<tr>
<td>Negative predictive value</td>
<td>1/4</td>
<td>25%</td>
<td>7/7</td>
<td>100%</td>
<td>6/7</td>
<td>85.71%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>3/12</td>
<td>25%</td>
<td>12/12</td>
<td>100%</td>
<td>10/12</td>
<td>83.33%</td>
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</table>

So, in case of adequate biopsies calretinin and synaptophysin confirms the results of H&E. While in inadequate biopsies calretinin and synaptophysin showed higher specificity (100%, 85.71%), sensitivity (100%, 80%) and accuracy (100%, 83.33%) respectively, compared to H&E results (specificity 14%, sensitivity 40%, accuracy 25%). The results of calretinin were superior to synaptophysin in diagnosis or exclusion of HD.

All enrolled specimens were examined by 2 pathologists, the measure of agreement by Kappa test showed there were high agreement in calreteinin and synaptophysin. Interestingly, the inter-rater agreement between two independent investigators is significant, reach about 100% in calreteinin (Kappa = 1), while for synaptophysin reaches 96.7% (Kappa = 0.916) and P value <0.001.

DISCUSSION

Hirschsprung disease (HD) is a genetically and phenotypically heterogeneous disorder characterized by complete functional obstruction and proximally dilated colon due to the absence of ganglion cells.9 The
aganglionic segment is due to failure of migration of neural crest cells (precursors of enteric ganglion cells) during organogenesis. The diagnosis of HD is usually based on a combination of the presenting symptoms, radiological studies, rectal manometry and histological features of rectal biopsy.

Despite the importance of using H&E and rectal biopsy in the diagnosis of HD, detection of ganglion cells in H&E sections can be a difficult process for the pathologist. Accordingly, immunohistochemical (IHC) stains are used to confirm the diagnosis of HD by some pathologists. Recently, several reports have described the use of calretinin and synaptophysin IHC in determining the characteristics of neural distribution in HD. Calretinin was not expressed in aganglionic segments of HD and was associated with nerve fibers whereas both ganglion cells and nerve fibers were immunopositive in normal colons, and was the preferred marker in identification of ganglion cells. Calretinin interpretation was much easier compared with acetylcholinesterase for the junior pathologist. “Calretinin IHC overcomes most of the difficulties encountered using the combination of histology and acetylcholinesterase staining regarding the need for rapid biopsy and highly expensive costs of acetylcholinesterase staining.”

Present study also showed that results of the calretinin and synaptophysin IHC in identification of ganglion cells and nerve fibers are superior to H&E staining results. In this study, results of calretinin IHC in rule out of HD were consistent with the results of H&E staining in adequate specimens. However, in inadequate samples calretinin and synaptophysin were more accurate in diagnosis and exclusion of HD when compared with H&E stained sections. Present study is consistent with the results of Gonzalo DH et al, study which was carried on Cleveland Clinic, Ohio.

Positive calretinin immunostaining was seen in ganglion cells, and nerve fibers also represents the presence of related ganglion cells. Finally, comparing the values of specificity and accuracy between calretinin and standard histology (H&E) showed that calretinin IHC presented significantly higher specificity and accuracy values than H&E staining specially in inadequate samples. Interestingly, high specificity, sensitivity and accuracy of calretinin in comparison with synaptophysin in detection of ganglion in inadequate sample. While use of calretinin immunohistochemistry in evaluation for HD appears to be increasing, the published peer-reviewed scientific literature of this technique remains relatively sparse, and a recent standard textbook of Pediatric Pathology comments that this technique may indeed represent a valuable diagnostic adjunct if the utility of the calretinin staining pattern can be confirmed by other authors.

CONCLUSION

Calretinin IHC is a very reliable adjunctive test in identification of ganglion cells and nerve fibers and consequently in ruling out of HD. Results of calretinin and synaptophysin are confirmatory in case of adequate biopsies, however in case of inadequate biopsies calretinin and synaptophysin are conclusive and diagnostic compared to H&E. Calretinin shows higher accuracy than synaptophysin in inadequate biopsies. In addition, calretinin IHC overcomes most of the difficulties encountered using histology of H&E and acetylcholinesterase. Although limitation of this study included the small population, the results trigger the needs for more investigations in this area. Using calretinin IHC in protocol of HD diagnosis is recommended according to our experience in our institute.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Ethics Committee of Menoufia University-Faculty of Medicine’s.
REFERENCES