Distribution of Ganglion Cells in Rectal Mucosa and Submucosa Fragments for the Diagnosis of Hirschsprung’s Disease

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Received Date: 24 February, 2022; Accepted Date: 07 March, 2022; Published Date: 10 March, 2022

Abstract

Background: Hirschsprung’s Disease (HD) is characterized by intestinal sub-occlusion and absence of enteric ganglion cells. The analysis of a fragment obtained by rectal biopsy confirms the diagnosis of HD. In a recent study, we demonstrated that the analysis of 60 sections per fragment of rectal mucosa and submucosa stained by HE may ensure a 90% diagnostic accuracy. In this study, in 54% of the cases, neurons were found in the deepest sections (50-60). This encouraged us to study their distribution in the rectal submucosa, as a means to simplify the diagnosis. Objectives: To develop a method that facilitates the diagnosis of HD by studying the distribution of ganglion cells in the submucosal plexus. To apply this method to samples from patients with suspected HD, in order to verify its accuracy. Methods: Using the calretinin technique, we studied the distribution of ganglion cells in 60 fragments of rectal submucosa from 19 cadavers received at our morgue (SVOC). After the study, the reading method created was used for diagnosis in 47 cases of suspected HD, using HE staining. The accuracy of the method was verified by comparing the results obtained with HE to those obtained with the acetylcholinesterase technique, considered as the golden standard in our laboratory. Results: The study of ganglion cell distribution showed that just by examining 15 of the 60 sections we could already diagnose HD with 93% accuracy. Conclusion: The study of ganglion cell distribution enabled the creation of a simplified method for reading the slides. The application of the method achieved good accuracy and it can be used as an alternative in the diagnosis of HD.

Keywords: Ganglion cells; Hirschsprung’s disease; Hematoxylin-eosin; Submucosa; Rectal biopsy

Abbreviations: HD: Hirschsprung Disease; SVOC -SP: Sao Paulo City Morgue; HE: Hematoxylin-Eosin; Ache: Acetylcholinesterase; ICF: Informed Consent Form

Background

Hirschsprung’s Disease (HD), also known as congenital megacolon, is characterized by intestinal sub-occlusion with an underlying pathology marked by the absence of neurons in the enteric nervous system, with involvement of varying segments of the large intestine [1]. The disease was first described by Harald Hirschsprung in 1886, but it was only much later (1948) that Whitehouse and Kernohan described its definite etiopathogenesis [1,2]. Rectal biopsy is one of the methods used to confirm the diagnosis of HD. Specimens are usually collected from the entire thickness of the rectal wall and the fragment obtained is
stained by the classic Hematoxylin-Eosin (HE) method. The identification of neurons in the myenteric plexuses rules out the diagnosis of HD. In HD, besides the absence of ganglion cells in the plexuses there is hypertrophy of nerve trunks [3,4]. Another histological method used in the diagnosis of the disease is the screening for Acetylcholinesterase (AChE) [5] activity, since the enzyme activity is typically increased in aganglionic segments of the intestine. Ganglion cells are believed to function as a growth sensor and in their absence, AChE-impregnated cholinergic nerve fibers undergo hypertrophy and hyperplasia, spreading towards the periphery. The patient’s mucosa and submucosa show large quantities of cholinergic fibers as compared to the normal intestine [6,7].

The AChE screening requires only a small fragment of mucosa and submucosa that can be obtained by suction biopsy. Therefore, this technique has an advantage over HE staining, not only because of its proven efficiency, but also because there is no need for a fragment of the entire bowel wall. The suction biopsy performed for this type of exam is much less invasive [8-10]. Nevertheless, the technique is not widely available, due to difficulties in processing the specimens and the use of reagents and equipment that make it more expensive than HE staining [10]. With the recent introduction of immunohistochemical markers, new method based on calretinin is being used to diagnose HD. Calretinin is a calcium-binding protein found in ganglion cells and thin fibrils in the lamina propria of patients with normal gut innervation. On the other hand, this protein is absent in patients with HD [11]. The calretinin immunohistochemical test requires only a small fragment of mucosa and submucosa, similar to the screening for AChE activity, and the fragment can be embedded in paraffin. However, immunohistochemical methods are more expensive and their execution is more complex when compared to HE staining [12].

The major disadvantage of HE staining would be the need for a full-thickness wall biopsy. Many authors still doubt the feasibility of a safe diagnosis of the disease through the analysis of nerve plexuses only in fragments of mucosa and submucosa, as neurons were found to be more sparse in this region, which could lead to misinterpretation of false-positives [10,13,14].

However, in a recently published study performed at our laboratory, we have shown that it is possible to have an accurate diagnosis of HD only with mucosal and submucosal fragments stained by HE, as long as at least 60 sections of each fragment are analyzed. In this same study, while reading the slides, we observed that ganglion cells were more easily located in the final sections (sections 50-60) in 54% of patients with normal gut innervation [15]. Using this method, we can accurately diagnose HD in a simpler and less invasive way. However, the need to analyze so many sections makes the process of reading the slides more time consuming. According to Qualman [16], the analysis of one single case by a pathologist would take about 30 to 60 minutes. These findings led to an interest in better studying the distribution of ganglion cells in the submucosal region of normal patients, since there are no pre-existing studies that assess enteric neurons in the submucosal region of healthy individuals. Based on this investigation, we decided to develop a model to systematize and facilitate the process of reading the slides for the pathologist, making it more efficient. It is worth mentioning that this study will be of great value for pediatric surgery services that have no access to such specific diagnostic methods for HD, such as the AChE and calretinin techniques.

**Methods**

This study was funded by FAPESP Project nº 2019/10339-1and has been approved by the Research Ethics Committee through the approval letter #3.119.706. We also obtained a letter of assent from the director of the São Paulo city morgue for the use of cadavers and a signed Informed Consent Form (ICF) from family members.

Study of The Distribution of Ganglion Cells in Intestines with Normal Innervation

Nineteen fragments from the rectal wall of cadavers received at the city morgue (SVOC-SP) between July 2019 and March 2020 were obtained with the consent of the respective family members. The exclusion criteria for the collection of specimens were cadavers with >12h post mortem, for quality reasons, and cadavers of ‘people with Chagas’ disease, which may cause acquired megacolon in adults due to the loss of ganglion cells in the enteric plexuses [17]. The specimens were collected by technicians and placed in 10% formaldehyde solution. After collection, the specimens were sent to the laboratory and a smaller fragment was obtained, measuring approximately 1 cm x 1 cm, from the posterior wall of the rectum, 2 cm above the pectineal line, simulating the procedure of rectal mucosa and submucosa biopsy performed in living patients. The specimen included the whole rectal wall, but for the study we analyzed only the mucosa and the submucosa. The tissue was dried and diaphonized in an automatic processor (Lupe, PT05, Brazil).

Using a microtome (Leica RM2255, Germany) sixty (60) 3 μm-thick sections were serially obtained from each fragment. We then applied the calretinin immunohistochemical technique using an antibody (Rabbit Anti-Human Calretinin Monoclonal Antibody - Clone SP13 - Abcam, USA) to identify the presence or absence of ganglion cells in all 60 sections of each specimen.

**Statistical Analysis**

The data from the slides were assessed and possible combinations (sets of sections) were generated through combinatorial analysis.
Each set of sections was tested for accuracy using the bootstrapping technique:

a. One thousand samples were randomly generated from the baseline material.
b. The accuracy (number of correct predictions divided by the number of samples) was calculated.
c. Processes a and b were repeated 100 times.
d. The accuracy of a set of sections was defined as the mean accuracy from 100 repetitions.

The 15 sections with the highest incidence among all the possibilities generated were selected.

We also carried out the study of the frequency of ganglion cells, verifying the accuracy according to the distance in microns by reading equidistant slices. The analysis was divided as follows.

a. Accuracy analyzing only the first cut - 0 µm distance
b. Accuracy analyzing the first and last cut - distance between cuts of 180 µm.
c. Accuracy analyzing the first, the 30th cut and the last cut - distance between cuts of 90 µm.
d. Accuracy analyzing the first, the 15th, 30th, 45th cut and the last one - distance between cuts of 45 µm.
e. Accuracy analyzing the first, the 8th, 15th, 22th, 30th, 37th, 45th, 52th cut and the last one - distance between cuts of 20 µm.

**Application of the Model to the Diagnosis of HD**

This retrospective study examined 47 paraffin blocks that were archived in our laboratory. These blocks were from biopsies obtained to confirm the diagnosis of HD in patients treated at our institution in the period 2016 - 2019. Two fragments of rectal mucosa and submucosa had been collected from each patient. One fragment had been frozen for the AChE activity screening and the other had been embedded in paraffin and stored for further studies. The fragment intended for AChE screening was processed by freezing and subsequently sectioned in a cryostat (Slee MEV, Germany). The acetylcholinesterase activity was demonstrated by the method of Karnovsky and Roots [7] and counterstained by Carrazi’s hematoxylin. The 47 specimens embedded in paraffin were sectioned with an electronic microtome (Leica RM2255, Germany). Sixty 3 µm serial sections were obtained from each specimen and processed by hematoxylin-eosin. The HE-stained slides were examined using the proposed reading method to identify the presence or absence of ganglion cells in the submucosal plexus; the observers had no information about the previous diagnosis made by AChE screening. The image below shows the submucosal plexus with two ganglion cells in evidence. 400x magnification (Figure 1).

![Submucosal plexus with two ganglion cells highlighted by the arrows. HE staining. 400x magnification.](image)

**Statistical Analysis**

To analyze the agreement between the HE staining and the AChE technique, i.e., the golden standard, we used the kappa and chi-square statistical model for sensitivity and specificity once the contingency tables were put together. The R software version 4.0.2 was used to perform all the analyses.

**Results**

**Study of Ganglion Cell Distribution In Samples with Normal Rectal Innervation**

All 60 sections of each fragment were analyzed. The results were recorded on a table showing which sections of each sample had ganglion cells present (Table 1). All possible combinations were generated using combinatorial analysis followed by the bootstrapping technique, to read the slides with satisfactory accuracy. With a total of 5 sections per set, 80,035 sets were generated where it would be possible to diagnose HD with an accuracy greater than 80%. As more than 80,000 possibilities were created to read the slides with satisfactory accuracy and it would not be possible to test all these possibilities, we chose for the formula the fifteen sections with most repetitions in all sets (selected sections: 4, 9, 10, 20 , 23, 26, 27, 32, 46, 47, 48, 51, 55, 56, and 59) (Table 2).
### Table 1: Presence of ganglion cells by cuts.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Section</th>
<th>Qty.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>295925</td>
<td>28%</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>277442</td>
<td>26%</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>227613</td>
<td>22%</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>208033</td>
<td>20%</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>203252</td>
<td>19%</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>191833</td>
<td>18%</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>188683</td>
<td>18%</td>
</tr>
<tr>
<td>8</td>
<td>55</td>
<td>186250</td>
<td>18%</td>
</tr>
<tr>
<td>9</td>
<td>51</td>
<td>183404</td>
<td>17%</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>180496</td>
<td>17%</td>
</tr>
<tr>
<td>11</td>
<td>32</td>
<td>177554</td>
<td>17%</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>176396</td>
<td>17%</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
<td>171363</td>
<td>16%</td>
</tr>
<tr>
<td>14</td>
<td>46</td>
<td>168304</td>
<td>16%</td>
</tr>
<tr>
<td>15</td>
<td>48</td>
<td>149700</td>
<td>14%</td>
</tr>
</tbody>
</table>

### Application of the systematization method to the reading of HE-stained specimens.

The biopsy specimens assessed in this study were received from the Children’s Institute at the University of São Paulo Medical School General Hospital (ICr-HCFMUSP). Of the 47 specimens, 31 were from children with HD and the other 16 were from children who did not have the disease based on the AChE screening. The samples were analyzed according to the formula created, and only the fifteen selected sections (4, 9, 10, 20, 23, 26, 27, 32, 46, 47, 48, 51, 55, 56, and 59) were used for diagnosis.

### Comparison between the AChE and the HE methods

The HE staining method was in agreement with the AChE method in 93.6% of cases, with a Kappa index of 0.850 (p-value <0.00001). Of the 3 discordant cases, 1 was a false positive and 2 were false negatives (Table 3). Significant accuracy, sensitivity and specificity values were also achieved (Table 4). The two cases considered as false negatives (cases 6 and 25) were analyzed with the calretinin immunohistochemical technique to verify if the visualized structures were really ganglion cells, and in both cases the calretinin marking was positive, demonstrating that the reading performed with the HE method was correct (Figure 2). The case considered as false positive was reassessed and in the new reading of the slides, a plexus with ganglion cells was found in section 4. In this case, the error was made by the observer (Figure 3).
Comparison AChE x HE

<table>
<thead>
<tr>
<th>AChE</th>
<th>HE</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>15</td>
<td>47</td>
</tr>
</tbody>
</table>

**Table 3:** Comparison between the diagnosis performed with the HE technique and with the AChE screening (golden standard). Negative means not ill and positive means presence of HD with both methods.

**Accuracy**

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>0.936</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.929</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.939</td>
</tr>
<tr>
<td>VPN</td>
<td>0.867</td>
</tr>
<tr>
<td>VPV</td>
<td>0.969</td>
</tr>
</tbody>
</table>

**Table 4:** Values for validation of the hematoxylin-eosin method.

**Figure 2:** Images of cases considered as false negatives by comparison with the AChE golden standard and assessed with the calretinin technique. Both with ganglion cell labeling. Figure a – case 6, 200x magnification. Figure b – case 25, 400x magnification.

**Figure 3:** Submucosal plexus image found in section 4 of case 8 that had been considered as false positive. HE staining. 400x magnification.

**Analysis of the frequency of ganglion cells by equidistant slices**

In the analysis of the frequency of ganglion cells by the distance in microns, we verified how many equidistant cuts were necessary to obtain an accuracy greater than 90%. It was observed that with 9 cuts, that is, analyzing the sample every 20 µm, an accurate diagnosis was possible (Table 5 and Figure 4).

**Accuracy by distance between cuts**

<table>
<thead>
<tr>
<th>Distance between cuts</th>
<th>Number of cuts</th>
<th>Cuts</th>
<th>Model accuracy</th>
<th>Validation accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µm</td>
<td>1</td>
<td>1</td>
<td>10,5%</td>
<td>69,6%</td>
</tr>
<tr>
<td>180 µm</td>
<td>2</td>
<td>1, 60</td>
<td>42,1%</td>
<td>76,1%</td>
</tr>
<tr>
<td>90 µm</td>
<td>3</td>
<td>1, 30, 60</td>
<td>52,6%</td>
<td>78,3%</td>
</tr>
<tr>
<td>45 µm</td>
<td>5</td>
<td>1, 15, 30, 45, 60</td>
<td>68,4%</td>
<td>80,4%</td>
</tr>
<tr>
<td>20 µm</td>
<td>9</td>
<td>1, 8, 15, 22, 30, 37, 45, 52, 60</td>
<td>100,0%</td>
<td>93,5%</td>
</tr>
</tbody>
</table>

**Table 5:** Values of accuracies according to the distance.
Discussion

Although the first description of HD was made in 1886 by Harald Hirschsprung, the disease still represents a challenge, both in terms of its etiopathogenesis and its genetic aspects [2,18-20]. After the pathophysiological characterization of HD in 1948 by Whitehouse and Kernohan, the rectal biopsy became the method of choice for confirming the diagnosis. However, there is still much controversy around the best technique for preparing the biopsy material [1,2,21]. Currently, the screening for AChE activity and the immunohistochemical marking with calretinin are the most widely used techniques to diagnose HD in fragments of rectal mucosa and submucosa [10-12,22]. However, new studies have emerged with the objective of simplifying the diagnosis using HE staining in these fragments of rectal mucosa and submucosa [8,13,15,16,23]. The use of HE staining to detect ganglion cells in the submucosal plexus and diagnose HD is a simple and fast method, but still the subject of controversy. Several authors mention the difficulty in identifying neurons in these plexuses, as they are diffusely distributed along the intestinal submucosa [16,24,25].

We demonstrated in a previous study [15] the possibility of circumventing the problem of neuron dispersion, as long as 60 sections of the same fragment were obtained for the evaluation of ganglion cells. During this study, ganglion cells were observed to be more frequent in the final sections of the fragment. This finding encouraged us to analyze the distribution of neurons in intestines with normal innervation. Our objective was to create a systematic procedure to read the slides that would simplify the diagnosis, reducing the pathologist’s workload, avoiding the need to analyze a large number of sections to obtain an accurate diagnosis. This systematization can speed up a process that usually takes, on average, 30 to 60 minutes if the pathologist has to read 60 sections [16,26]. The study of normal gut fragments was carried out in post-mortem material collected from adult donors due to the ease of obtaining the samples. It is proven that the difference between innervation in adults and children is only related to ganglion cell maturation, which would not interfere in our study [27-29]. We chose to use calretinin in these samples so that no ganglion cell would go unnoticed by the observer while reading the sections [12,22].

By analyzing the distribution of neurons in the fragments with normal innervation, we were able to choose only 15 sections from the total of 60. Using the bootstrapping statistical method, we verified that these 15 selected sections had a higher incidence of ganglion cells when compared to the other sections, therefore being representative of the whole specimen. When applying the systematization model to the 47 cases suspected of HD with specimens stained by HE, we read only these 15 sections and obtained a satisfactory 93% accuracy. These findings showed that the model achieves an accuracy similar to more specific methods, being valid for the diagnosis of the disease [10,11,15,22]. When comparing the results with HE to those with our golden standard for diagnosis (AChE) we found only one false positive, later identified as an observer error, i.e., there was a ganglion cell in section 4 - one of the cuts selected for the model [21,23]. There were also two cases in which neurons were visualized with HE staining, but the diagnosis made using the AChE technique showed acetylcholinesterase activity. These two cases were reviewed using calretinin and the presence of neurons was confirmed. In these cases, the biopsy was probably performed in the HD transition zone, a region that may exhibit both neurons and increased cholinesterase activity in fibrils and nerve trunks [30]. In the analysis of the frequency of ganglion cells by equidistant slices, we could demonstrate that there is a possibility of a diagnosis with satisfactory accuracy, provided that, within the 180 microns of the fragment, a slice is visualized every 20 microns. While we still consider specific methods as the best option for diagnosing HD, we believe the results of this study can offer a good alternative in situations where these methods are not available.

Conclusion

We conclude that the study of the distribution of ganglion cells in fragments of the rectum with normal innervation was useful to create two simplified methods for reading slides and diagnosing HD using HE staining. Using this reading method, the agreement between the AChE screening and the HE staining technique was satisfactory, offering an alternative for the diagnosis of the disease.

References


