

Video Article

Diagnosis of Hirschsprung's Disease by Immunostaining Rectal Suction Biopsies for Calretinin, S100 Protein and Protein Gene Product 9.5

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Abstract

Hirschsprung's disease (HD) is a congenital intestinal disease that is clinically manifested as an inability to pass meconium in infants or as long-term constipation in children. Rectal suction biopsy (RSB) to determine the absence of ganglion cells and neural hypertrophy is the most accurate test for the diagnosis of HD at present. Traditional hematoxylin-eosin staining lacks sensitivity and specificity. Acetylcholinesterase staining cannot be widely used due to its complex process. Our novel protocol of immunostaining for calretinin, S100 protein, and protein gene product 9.5 (PGP9.5), which we conducted on RSBs, exhibits high sensitivity and specificity rates of 96.49% (95% confidence interval, 0.88-0.99) and 100% (95% confidence interval, 0.97-1.00), respectively. The HD-affected segments often present as the absence of the expression of calretinin, S100 protein, and PGP9.5, which are markers of neural hypertrophy in the submucosal tissue. This protocol describes the detailed operating process of this new diagnostic method.

Introduction

Hirschsprung's disease (HD) is a common congenital gut intestinal disorder characterized by a lack of ganglion cells in different segments of the distal intestinal tract¹. The human enteric nervous system is formed when the invasion of the embryonic neural cells is completed. If there is a disturbance of the process and the invasion fails to complete, the distal intestine of the newborn becomes aganglionic². This potentially fatal condition is called Hirschsprung's disease. Proliferation, motility, and gut growth are the three main components of successful colonization.

Traditional hematoxylin and eosin (H&E) staining of a limited submucosal biopsy cannot attain as satisfactory a result as H&E staining of a full-thickness tissue obtained from surgery. Additionally, acetylcholinesterase (AChE) staining of rectal suction tissue is theoretically challenging due to its inadequate sensitivity, which is 91%, and complex processing of frozen sections^{3,4}. Several other immunohistochemical markers of ganglion cells and nerve fibers that can be stained in formalin-fixed and paraffin-embedded specimens are gradually becoming the mainstream HD diagnostics. Calretinin is a vitamin D-dependent calcium-binding protein that is not expressed in the myenteric and submucosal plexus of HD-affected segments⁵. S100 protein is expressed in cells derived from the neural crest, such as nerve fibers and glial cells, which often present neural hypertrophy in the submucosal tissue of HD-affected segments⁶. Protein gene product 9.5 (PGP9.5) reliably stains nerve fibers and ganglion cells; PGP9.5 staining acts as a supplement to calretinin staining, especially in cases of isolated hypoganglionosis. Double staining with S100 and PGP9.5 can decrease the false-negative rate and increase the sensitivity. As a prerequisite, the current study aims to ensure adequate specificity and high sensitivity of this novel diagnostic method. Our novel protocol used all three markers for the discrimination of aganglionic intestine and hypertrophic nerve fibers. A prospective study of 318 children was performed by our lab and previously published without a detailed protocol⁷. The detailed protocol and precautions are discussed in this article. Any neonates who suffered from a severe defecation problem since birth or children with chronic constipation excluding other common diseases are potential candidates for rectal suction biopsy (RSB). Our novel protocol is suitable for staining not only RSBs but also full-thickness biopsies or surgical specimens to make a final diagnosis.

Protocol

This protocol was approved by the Research Ethics Board of the Union Hospital of Huazhong University of Science and Technology.

1. Rectal Suction Biopsy

1. Perform the rectal suction biopsy by a well-trained pediatric surgeon and an assistant using a Rbi2 suction rectal biopsy system after obtaining informed consent from the guardian.
 1. Perform RSB on patients that have the following indications: inability to pass meconium in infants or long-term bloating with or without constipation in children; children with long-term constipation who need paraffin oil to complete defecation; intestinal obstruction or intestinal perforation with unknown reasons in children undergoing an enterostomy.

NOTE: The contraindications for RSB are as follows: children who have severe symptoms, who are in poor physical condition, or who cannot tolerate the RSB; children with acute-stage enterocolitis; children undergoing intestinal anastomosis without complete healing.

2. Perform a normal saline retention enema as bowel preparation 36 h before the procedure to avoid loose stools and excessive edema of the mucosa. Add magnesium sulfate solution if the child has severe constipation. Administer vitamin K (1 mg/kg) to neonates.
NOTE: Do not perform preoperative blood tests or administer antibiotics, as they are not required.
3. Put patient in the lithotomy position. Coat the surface of the tube with paraffin oil. Insert the blunt-ended tube 4-7 cm into the rectum with the side hole facing the posterior or lateral walls.
4. Apply gentle pressure on the tube to facilitate adequate adhesion of the side hole to the rectal wall. Press the trigger and withdraw the tool immediately.
5. Use the biopsy instrument to obtain 4 suction biopsies with a diameter of 2 mm at 3 cm and 6 cm above the dental line, anteriorly and posteriorly. Use a needle to place the specimen on gauze moistened with saline.
6. Observe the patient until 2 h after the procedure to rule out rectal bleeding. Avoid further rectal and anal manipulations in the first 24 h after RSB.

2. Preparation of the RSB Sections

1. Place the rectal suction biopsies in 4% paraformaldehyde for 6 h to fix the tissue. Use a fixative volume 5 times more than the tissue volume.
2. Dehydrate the tissue using the pathological tissue dehydrator for 11 h. The whole programmed process consists of the following 5 steps:
 1. Place the tissue in formaldehyde and fix for 1 h.
 2. Place the tissue in 75% ethanol for 4 h.
 3. Place the tissue in absolute ethanol (two changes, 1 h each).
 4. Place the tissue in absolute ethanol (two changes, 30 min each).
 5. Place the tissue in xylene (three changes, 1 h each).
3. Place the tissue in paraffin wax (58-60 °C) (three changes, 1 h each). Embed the RSB tissues in paraffin blocks.
4. Trim the paraffin blocks using a microtome until the tissue is entirely exposed with a complete section plane.
5. Cut the blocks into 0.4 µm sections with a microtome.
6. Place the sections in 30% ethanol first and then into 45-50 °C water for a few seconds each to allow the sections to spread into a flat plate.
7. Transfer the paraffin sections onto slides.
8. Bake the slides in a 60 °C oven for 3-5 h. Avoid baking for too long, which may lead to the loss of the antigens.
9. Place the deparaffinized slides into xylene (2 changes, 15 min each).
10. Hydrate the slides in absolute ethanol, 95%, 80%, and 75% ethanol for 5 min each. Then, rinse them in reverse osmosis (RO)-purified water for another 5 min.

3. Antigen Retrieval

1. Make working dilutions for calretinin retrieval by mixing 4 mL of EDTA antigen retrieval buffer with 200 mL of RO-purified water. Make working dilutions for S100 and PGP9.5 retrieval by mixing 1 mL of citric acid sodium citrate buffer (100x) with 99 mL of RO-purified water.
NOTE: Calretinin can be retrieved preferentially by EDTA retrieval solution. However, citric acid sodium citrate buffer is more suitable for the retrieval of S100 and PGP9.5.
2. Place the slides in a coplin staining jar. Fill the jar with retrieval solution and place it into a preheated boiling water bath. After boiling for 20 min, remove the jar from the water bath and let it cool to room temperature. The cooling process takes approximately 10 min. Remove the slides and gently rinse them once with phosphate-buffered saline (PBS).
3. Draw a circle with a marker pen around the tissue and prepare a wet box for the following procedures.

4. Blocking

1. Make working dilutions by mixing 3 mL of 30% H₂O₂ with 27 mL of RO-purified water.
2. Add two or three drops of 3% H₂O₂ solution dropwise onto the tissue to block peroxidases. Add the H₂O₂ solution promptly to ensure that the solution will not overflow from the circle. Perform blocking of peroxidases in a 37 °C incubator for 20 min.
3. Rinse the slides gently with PBS (three washes, 5 min each).
4. Block the slides for 20 min at 37 °C with 5% bovine serum albumin (BSA, prepared by mixing 1.5 g of BSA powder with 30 mL of RO-purified water).
NOTE: Blocking with 3% H₂O₂ can prevent 95% of nonspecific staining and blocking with 5% BSA can prevent the other 5% of nonspecific staining. Combine the two methods to obtain a reliable result.

5. Antigen-antibody Reaction

1. Remove the 5% BSA and add 50 µL of primary antibody (calretinin, S100, or PGP9.5) to the slide. Incubate overnight at 4 °C.
2. Wash the slide with PBS (three times, 3 min each).
3. Add 50 µL of polymer enhancer (reagent A; see **Table of Materials**) to the section and incubate it for 20 min in a 37 °C incubator. Then, wash the slide with PBS (three times, 3 min each).
4. Add 50 µL of secondary antibody B (goat anti-rabbit/mouse IgG secondary antibody) to the section and incubate for 30 min in a 37 °C incubator. Wash the section with PBS (three times, 5 min each).

6. 3,3-Diaminobenzidine and Hematoxylin Staining

1. Add 850 μ L of RO-purified water to a 1.5 mL tube and add the staining reagents (see **Table of Materials**) in the order A, B, and C. Add 50 μ L of each reagent to obtain a total of 1 mL of 3,3-diaminobenzidine (DAB) working solution. If there are precipitates, use the solution after filtration.
2. Add a drop of DAB working solution to the tissue section and stain for 3-10 min. Incubate the slides in DAB at room temperature until brown staining is detected. Monitor carefully using a brightfield microscope. Keep the DAB working solution away from light. Then, wash the slide with PBS for 5 min.
3. Add a drop of hematoxylin to counterstain the nuclei for approximately 1 min. Then, hold the coplin jar and wash it under a trickle of water for approximately 30-40 s until the excess dye is removed. Be careful not to damage the tissue sections.
4. Immerse the RSB slides in a coplin jar containing PBS for 25-30 s. Then, differentiate in 1% hydrochloric acid-ethanol for 10 s. Wash the slides with PBS for 1 min.
5. Dehydrate the slides in the reverse order of hydration: 75%, 80%, 90%, and absolute ethanol (5 min each).
6. Expose the slides to xylene (2 changes, 5 min each).
7. Mount the coverslip using ultraclean mounting. Allow slides to dry before visualization using a microscope.

Representative Results

In total, 318 patients were enrolled in our study. All of the patients underwent RSB, and the tissues were stained for calretinin, S100, and PGP9.5. The diagnosis based on our novel protocol was HD in 97 cases, non-HD in 213 cases, and suspected HD in 8 cases. Among the 132 surgical patients, 99 patients were diagnosed with HD by immunostaining of full-thickness specimens after surgery. S100 and PGP9.5 staining showed that 92% and 93% of the 99 patients, respectively, had hypertrophied nerve trunks. The other 83 of the 186 patients underwent full-thickness biopsies and showed ganglion cells. A total of 103 patients were clinically cured by conservative therapies, and HD was excluded. Of the 8 patients with suspected HD, 3 patients were categorized as short-segment HD, and the other 5 patients were categorized as non-HD.

According to our results, 97 patients were diagnosed by RSB initially. Detailed information is shown in **Table 1**. The protocol of immunohistochemical staining for calretinin, S100, and PGP9.5, which we conducted on RSBs, has high sensitivity and specificity rates of 96.49% (95% confidence interval [CI], 0.88-0.99) and 100% (95% CI, 0.97-1.00), respectively. The positive predictive value is 94.3% (95% CI, 0.87-0.99), and the negative predictive value is 99.1% (95% CI, 0.95-1.00).

Figure 1A and **1B** represent typical results after immunostaining for calretinin. Many ganglion cells, which express calretinin, can be found in the normal tissues. However, no ganglion cells were detected in any area of the tissue from the HD segment. As shown in **Figure 1C-F**, S100 and PGP9.5 immunostaining shows hypertrophied nerve trunks in the submucosa, while only granular staining of some small nerve twigs was observed in the normally innervated intestine. In our study, hypertrophic nerve trunks are nerve fibers with a diameter of $\geq 40 \mu\text{m}$.

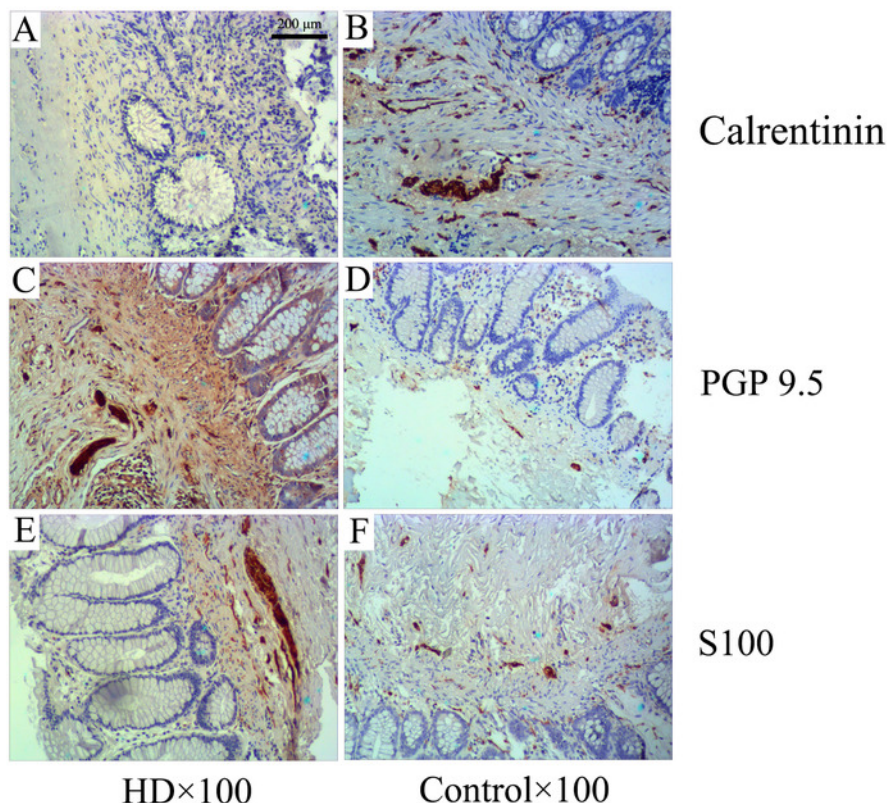


Figure 1: Immunostaining of rectal suction biopsies for calretinin, PGP9.5, and S100. Immunostaining for calretinin in HD-affected tissue (A) and in ganglion cells of myenteric plexuses in normally innervated tissue (B). No calretinin staining was detected in the myenteric plexuses of the HD-affected segment. PGP9.5 staining in HD-affected tissue showed dense hypertrophic nerve fibers in the submucosa (C) in contrast to nerve fibers in the normal tissues (D). (E) Dense and prominent S100 immunostaining showed hypertrophic nerve trunks in the submucosa of HD-affected tissue. (F) Normal nerve fibers in the submucosa. [Please click here to view a larger version of this figure.](#)

protein markers	HD(99)		Non-HD(219)	
	ganglion cells (+)	Hypertrophic nerve trunks (+)	ganglion cells (+)	Hypertrophic nerve trunks (+)
calretinin	2	—	214	—
S100	—	91	—	3
PGP9.5	—	92	—	4

Table 1: Immunohistochemical staining scores for the three protein markers in rectal suction biopsies.

Discussion

Here we described a procedure using three different immunohistochemical antibodies to stain RBS sections for the diagnosis of HD. The sensitivity of our diagnostic protocol was 96.49% (95% CI, 0.88-0.99), and the specificity was 100% (95% CI, 0.97-1.00).

The most critical steps of the protocol are RSB and antigen-antibody reaction. The size of the biopsy determines the accuracy of the staining. A small biopsy will not provide enough tissue to make a precise diagnosis, while a large biopsy leads to a higher risk of bleeding. As a matter of experience, biopsies with a diameter of 2 mm are the optimal size. For the antigen-antibody reaction, proper preservation of the antibody cannot be ignored. Following the protocol step by step and gently working are needed. Reverse osmosis-purified water is recommended in all steps, yielding a clearer field for observation and making it easier for pathologists to make an accurate diagnosis.

Contrast enema, anorectal manometry, and biopsy with histology are three preoperative diagnostic methods that have been used in recent years. According to Takawira *et al.*'s research, biopsy with histology has the highest sensitivity and specificity⁸. Meier-Ruge *et al.* first reported the use of acetylcholinesterase (AChE) staining on frozen rectal suction tissue to help diagnose HD in 1972⁹. This staining method detects hypertrophic nerve trunks in the submucosa. After that report, many institutions around the world started to use this method as an adjunct for the diagnosis of HD. Due to its diagnostic limitations, researchers started to concentrate on other markers that can also stain the ganglion cells and nerve fibers in formalin-fixed and paraffin-embedded specimens. In 2004, Barshack *et al.* found that the loss of calretinin expression may help diagnose aganglionic segments in HD¹⁰. Kapur *et al.* and Guinard-Samuel *et al.* repeated the protocol in 2009 and concluded that immunohistochemical staining for calretinin was superior to staining for acetylcholinesterase^{11,12}. In 1988, S100 immunostaining for the diagnosis of HD was first introduced by Robey *et al.*¹³. Monforte-Muñoz *et al.* repeated the method in 1998 and found that 90% of their 20 Hirschsprung cases had

nerve fibers wider than $40\text{ }\mu\text{m}$ ¹⁴. Therefore, S100 immunostaining may serve as an ancillary diagnostic method in cases without ganglion cells. Furthermore, Bachmann *et al.* noticed that immunohistochemical staining for MAP2, calretinin, GLUT1, and S100 could obtain a reliable result as accurate as frozen section techniques¹⁵. However, in 1992, Sams *et al.* evaluated the value of PGP9.5 in the diagnosis of HD¹⁶. Although S100 can detect more lamina propria nerve fibers, it has a higher false-negative rate. Therefore, we chose to use PGP9.5 and S100 together for the immunohistochemical diagnosis of HD, as previous studies recommended. Many cases of staining for neuron-specific enolase in transitional zones also showed positive ganglion cells in the myenteric and submucosal plexus, but these cells were sparse and small¹². Similarly, calretinin-positive ganglion cells are small and have a clustered formation. A previous study indicated that some slightly thickened nerve trunks could also be detected in the transitional segments of HD, but with total colonic aganglionosis, the nerve trunks may be within normal limits ($< 40\text{ }\mu\text{m}$)¹⁷. We should analyze the results case by case and combine these results with clinical manifestations to avoid misdiagnosis.

This protocol could not represent the final pathological diagnosis, so we carried out a minimum of one year of thorough clinical follow-up for each child. Additionally, this protocol requires more samples to prove its reliability.

In conclusion, the diagnosis of HD by RSB is a convenient method that provides an ideal histological diagnosis with acceptable minimal injury. The sensitivity is closely related to whether the site of sampling is correct. Immunohistochemical staining of RSB for calretinin, S100, and PGP9.5 is sensitive and specific in the detection of ganglion cells and hypertrophic nerve trunks. The combination of these three markers offers a more reliable diagnostic method for HD.

Disclosures

The authors have nothing to disclose.

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