

Application of Acetylcholinesterase Histochemistry for the Diagnosis of Hirschsprung's Disease in Neonates and Infants: a Twenty-year Experience

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Key words: acetylcholinesterase; rectal biopsy; Hirschsprung's disease; neonates; infants.

Summary. Background. The acetylcholinesterase (AChE) histochemical staining of intestinal mucosal-submucosal biopsy specimens is believed to be the most reliable diagnostic method for Hirschsprung's disease (HD). The aim of our study was to evaluate advantages and disadvantages of this method for HD diagnosis in infants and neonates.

Material and Methods. The results of AChE histochemistry of rectal biopsy specimens, obtained from 11 neonates and 29 infants treated in the Clinic of Pediatric Surgery, Hospital of the Lithuanian University of Health Sciences, from 1991 to 2010 were analyzed. AChE activity of neural structures was evaluated using Karnovsky–Roots method modified by El-Badawi and Schenk.

Results. Two neonates were diagnosed with HD. The diagnosis was not confirmed in 9 cases, but clinical symptoms progressed in 3 cases, and HD was diagnosed after the repeated biopsy performed in infancy. The results of primary biopsy were rated as false negative. Test sensitivity and specificity in neonates were 40.0% and 100%, respectively. A total of 21 infants were diagnosed with HD. All of them underwent surgery. The diagnosis of HD was confirmed in 20 cases; in one case, intestinal neuronal dysplasia type B was diagnosed. The diagnosis was not confirmed in 8 cases. In infants, the test had a sensitivity of 100% and a specificity of 88.8%.

Conclusions. The analysis of AChE activity in rectal biopsy specimens is a reliable method for diagnosing HD in infancy. This test is less valuable in neonates. If test results are negative, infants should be observed, and if symptoms persist, the biopsy should be repeated at the age of 3 months. Rectal biopsy specimens in neonates should include mucosa and submucosa.

Introduction

Hirschsprung's disease (HD) is a congenital absence of ganglion cells in the enteric plexuses of the large intestine (aganglionosis), manifesting as acute or chronic bowel obstruction. In 70%–90% of cases, clinical symptoms are present on the first days after birth. Hirschsprung's disease should be suspected in case of delayed meconium elimination, bloating, vomiting, and impaired defecation of the newborn (1–4).

The diagnosis of HD should be established early in the neonatal period, because without an effective diagnosis and appropriate treatment, a considerable proportion of infants will develop serious complications, such as acute enterocolitis or toxic megacolon (3).

With the advances in neonatal anesthesiology and surgical care, the use of the primary transanal endorectal pull-through procedure in the management of neonates with HD represents a significant change from the classical approach to its treatment (1).

However, HD diagnostics in the neonatal period remains a challenge. The most popular noninvasive

methods for diagnosing HD – radiocontrast enema or anorectal manometry – do not provide sufficient diagnostic information in neonates (1–3, 5).

Since the development of rectal suction biopsy technique by Noblett (1969) and the introduction of acetylcholinesterase (AChE) histochemistry by Meier-Ruge et al. (1972), which have demonstrated that the absence of ganglion cells in Meissner's plexus and increased AChE activity of neural structures in lamina propria mucosae are specific to Hirschsprung's disease, AChE histochemical staining of rectal suction biopsies has gradually replaced conventional full-thickness biopsy at many institutions (6–8). The aim of this study was to determine the value of AChE staining for rectal biopsy specimens in the diagnosis of Hirschsprung's disease in neonates and infants.

Material and Methods

The results of AChE histochemical staining in rectal biopsy specimens obtained from 40 neonates and infants treated in the Clinic of Pediatric

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Surgery, Hospital of the Lithuanian University of Health Sciences, from 1991 to 2010 were retrospectively analyzed. Indications for rectal biopsy were clinical, sonographic, and radiological signs of Hirschsprung's disease (constipation since birth, distended abdomen, a caliber change between a small or normal-sized distal aganglionic segment and a dilated proximal ganglionic bowel on ultrasound or x-ray examinations) or a colostomy due to low intestinal obstruction in the neonatal period. Eleven neonates aged from 11 days to 4 weeks (mean age, 18 days) and 29 infants aged 1–10 months (mean age, 4 months) were examined.

Before the biopsy procedure, children had one cleansing enema in the evening and one in the morning. Rectal biopsies containing mucosa and submucosa were taken using endoscopic forceps through the endoscope at 5 and 3 cm above the dentate line (linea anorectalis) and excised with surgical scissors using a rectal speculum at 1 cm above the dentate line. All three biopsy specimens in neonates were acquired using rectoscopic biopsy forceps. Biopsy was performed under anesthesia. No profuse bleeding from the biopsy places was identified after the procedure.

All the samples were frozen immediately by liquid nitrogen or ice and cut in a cryostat at temperature -25°C in serial sections of $15\ \mu\text{m}$. AChE staining was performed according to the method of Karnovsky and Roots, modified by El-Badawi and Schenk (9). The sections were fixed in 4°C 4% neutral formalin, rinsed for 1 minute in two portions of distilled water, and placed in the incubation medium consisting of the following: acetylthiocholine iodide, 12.5 mg; 0.06 N sodium acetate, 15.8 mL; 0.1 N acetic acid, 0.5 mL; 0.1 M sodium citrate, 1.2 mL; 30 mM copper sulfate, 2.5 mL; 4 mM iso-OMPA, 0.5 mL; and 5 mM potassium ferrocyanide, 2.5 mL. Incubation time was 120 minutes in a thermostat at 37°C . pH of the incubation medium was 5.5–5.6. Afterwards, the specimens were rinsed in distilled water, dehydrated for 30 s in 95% alcohol and for 30 seconds in absolute ethanol, clarified in 3 portions of xylol, 1 minute each, and coated. One section of the specimens after incubation was rinsed in the distilled water, kept for 30 seconds in Harry hematoxylin, then 1 minute rinsed in distilled water, faded in 1% lithium carbonate, rinsed for 1 minute in distilled water, dehydrated, and coated.

AChE reaction in the mucosal layer was evaluated according to these features:

1. Negative AChE reaction – no AChE-positive nerve fibers in the lamina propria mucosae and muscularis mucosae (Fig. 1A).
2. Slightly positive AChE reaction – AChE-positive nerve fibers in the muscularis mucosae and some fibers running transversely to the lamina propria mucosae (Fig. 1B).

3. Positive AChE reaction – a thick network of AChE positive nerve fibers in the muscularis mucosae and lamina propria mucosae (Fig. 1C).

The presence or absence of submucosal ganglia was also evaluated.

According to the pathologists' consensus (10), Hirschsprung's disease was diagnosed when the following findings were present in all biopsy specimens:

1. Positive AChE reaction in mucosal nervous structures;
2. Profuse submucosal nervous plexuses with high AChE staining intensity;
3. No ganglia in the submucosa.

A total of 120 biopsy specimens were examined. Test results were compared between the groups of neonates and infants.

The structure of nerve ganglia and neuronal cells using Cresyl fast violet staining was evaluated (11). After the fixation with Carnoy's solution, $7\text{-}\mu\text{m}$ paraffin sections were prepared and stained with cresyl fast violet solution (cresyl fast violet, 0.5 g; distilled water, 100 mL). Tissue differentiation was performed with glacial acetic acid solution (glacial acetic acid, $250\ \mu\text{L}$; alcohol, 100 mL). The sections were covered with Roti[®]-Histokitt II mounting medium.

Statistical Analysis. Sensitivity and specificity of this test in two groups of patients – neonates and infants – were calculated. Sensitivity measures the proportion of actual positives, which are correctly identified as such (e.g., the percentage of people with disease who are correctly identified as having the condition; sensitivity = true positives/true positives + false negatives). Specificity measures the proportion of negatives, which are correctly identified (e.g., the percentage of healthy people who are correctly identified as not having the condition; specificity = true negatives/true negatives + false positives).

Results

All 120 biopsy specimens excised with forceps and scissors included mucosa and submucosa.

Two neonates had a positive AChE reaction in the nervous structures of the mucous membrane and no ganglia cells in the submucosa – HD was diagnosed (Fig. 2A). They underwent radical operations and were confirmed to have Hirschsprung's disease pathologically. Six neonates had a negative AChE reaction in the mucous membrane, and ganglia cells were present in the submucosa (Fig. 2B). The diagnosis of HD was excluded. As these children matured, clinical signs of constipation regressed. Three neonates did not have ganglia cells in the submucosa, but they also had a negative AChE reaction in the nervous structures of the mucosa (Fig. 2C). HD was excluded. As these neonates presented with progressing clinical symptoms, biop-

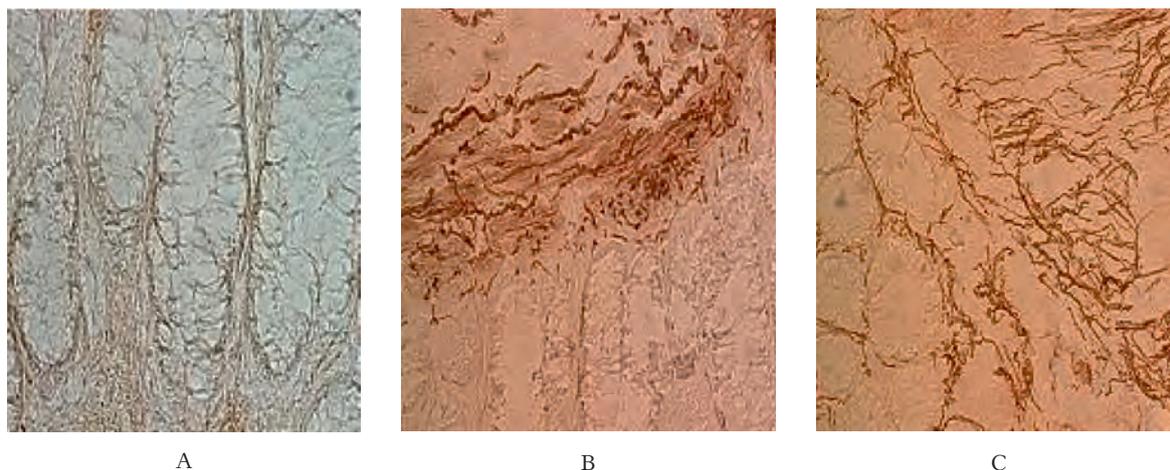


Fig. 1. Acetylcholinesterase (AChE) reactions in the mucosal layer

A, there are no AChE-positive nerve fibers in the lamina propria mucosae and muscularis mucosae that indicates a negative AChE reaction ($\times 40$); B, AChE-positive nerve fibers in the muscularis mucosae and some fibers in the lamina propria mucosae that indicates a slightly positive AChE reaction ($\times 20$); and C, a thick network of AChE-positive nerve fibers in the muscularis mucosae and lamina propria mucosae indicating a positive AChE reaction ($\times 40$).

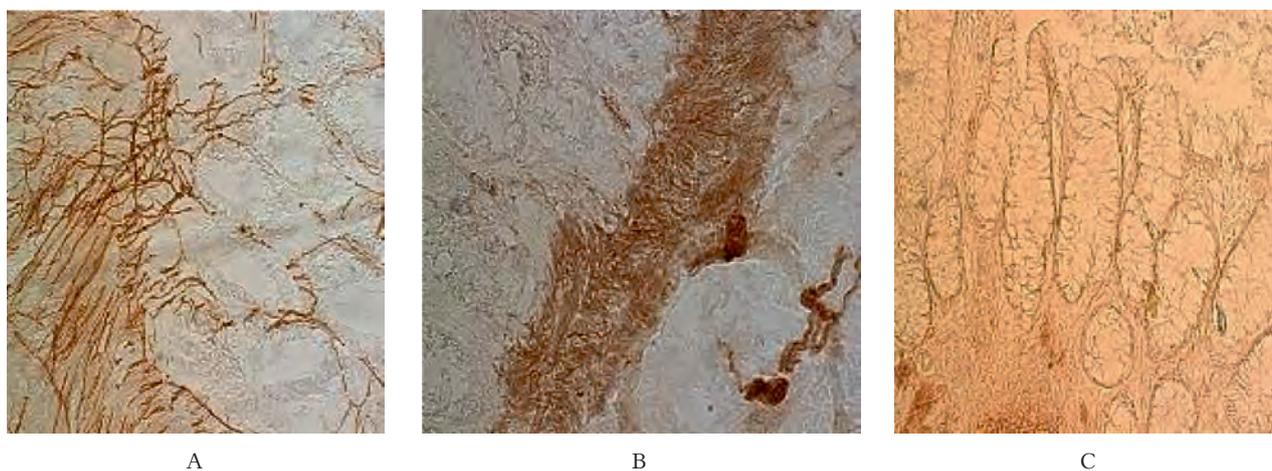


Fig. 2. Acetylcholinesterase (AChE) staining in various clinical cases

A, profuse nervous plexuses in the muscularis and lamina propria mucosae indicating Hirschsprung's disease ($\times 40$); B, no AChE-positive nerve fibers in the muscularis and lamina propria mucosae are observed; AChE-positive nerve ganglia in the submucosa indicates a normal case; diagnosis of HD was not confirmed ($\times 20$); and C, the proliferation of AChE-positive fibers is not seen in lamina propria mucosae indicating false-negative Hirschsprung's disease ($\times 40$).

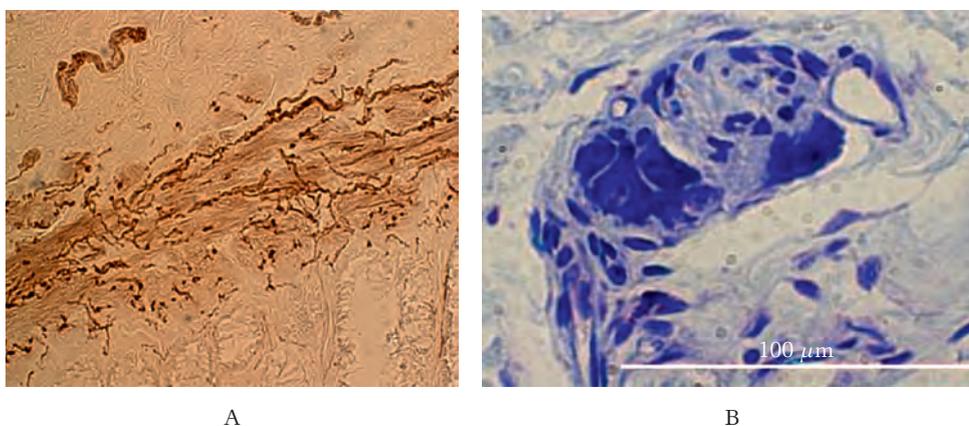


Fig. 3. Specimens from a patient with intestinal neuronal dysplasia

A, AChE-positive nerve ganglia in the submucosa and the proliferation of AChE-positive nerve fibers in the muscularis mucosae and some nerve fibers in the lamina propria mucosae ($\times 40$), B, the nerve cells in a ganglion of the submucosa stained with cresyl fast violet.

sies were repeated at the age of 3 months. Neuromorphological features of HD were detected. These infants underwent surgery, and the diagnosis of HD was confirmed pathologically. The results of primary biopsy were rated as false negative. In neonates, the sensitivity and specificity of the AChE staining for mucosal-submucosal rectal biopsies in establishing the diagnosis of HD were 40.0% (of the 5 cases, 2 positive and 3 false negative results) and 100% (of the 6 cases, 6 true negative results), respectively.

Neuromorphological features characteristic of HD were determined in 20 infants. All the patients underwent radical operations and were confirmed to have Hirschsprung's disease pathologically. In one case, an infant who had a colostomy for the low bowel obstruction at the age of 3 days, all biopsy specimens had a positive AChE reaction. During the surgery, a segment of the colon was removed, and after neurohistological and neurohistochemical examination of this segment, neuronal ganglia were found in the submucosa (Fig. 3). Intestinal neuronal dysplasia type B (IND B) was diagnosed. The results of primary biopsy were rated as false positive. Eight infants had a negative AChE reaction, and the diagnosis of HD was not confirmed. All infants received conservative treatment and their condition improved. In infants, the sensitivity and specificity of the AChE staining for mucosal-submucosal rectal biopsies in establishing the diagnosis of HD was 100.0% (of 20 cases, 20 true positive results) and 88.8% (8 true negative and 1 false positive result), respectively.

Discussion

Most authors report that AChE staining of rectal mucosal biopsy specimens is essential for a definite diagnosis of HD, especially when barium enema and anorectal manometry tests are not informative (due to total colonic aganglionosis, short-segment HD, neonatal age, or presence of a colostome) (3, 5, 7, 8, 12, 13). HD is characterized by profuse nervous plexuses in the submucosa, muscularis mucosae, and nerve fibers running transversely and forming a network of thin terminal nervous fibers in the lamina propria mucosae. All these nervous plexuses have very intense AChE activity. There are no ganglia in the submucosa (10, 12). False-positive results are often obtained when hemorrhagic specimens are being analyzed, because of high concentration of AChE in the red blood cell membrane (13). Possible causes for false-negative test results include variability in the biopsy site, too superficially taken biopsy material that lacks muscularis mucosa, immaturity of the enzyme system, technical variations in performance of staining, and the experience of individual pathologists (6). False-negative results are firstly associated with the patient's age (5, 13, 14). Chow et al. reported a type B positive reaction in

neonates, when only isolated AChE-positive fibers were detected in the lamina propria mucosae, and most of these fibers were located in muscularis mucosae and submucosa (15). This phenomenon was also observed by other authors, but they reported that such AChE reaction was not sufficient to confirm HD diagnosis (3, 14). AChE activity characteristic of classical HD was observed in only 83% of infants aged less than 3 months (14). Ganglia cells in neonates may be immature or located sparsely, thus imitating aganglionosis (16). Morphological alterations characteristic of HD are detected only in the distal parts of the colon below the lienal flexure, because this part of the large bowel receives parasympathetic innervation from the medullar S2–S4 segments. Therefore, AChE staining of the biopsy specimens taken from the ascending and transversal colon is not informative. This explains false-negative results of AChE staining in children with total colonic aganglionosis (10, 12–14). In our study, 1 false-positive result (2.4%) in an infant with neuronal intestinal dysplasia was documented, when ganglia in the submucosa were evaluated inaccurately. Three false-negative results were obtained after biopsies in neonates. Such results might be influenced by inadequate fiber proliferation in mucous membrane during the first weeks after birth. There were no cases of total aganglionosis in our sample. When biopsies were repeated after 3 months, positive AChE reaction was observed in the mucosal plexuses. These repeated biopsies confirmed HD; therefore, there was no need for full-thickness biopsies. This suggests that if the results of primary biopsy are negative, infants should be further observed. If symptoms persist, biopsy should be repeated at the age of 3 months (5).

Many different methods were proposed for the evaluation and diagnosis of congenital defects of the autonomic nervous system in the large intestines: histological, immunohistochemical (neuropeptide Y, substance P, tyrosine hydroxylase etc.), neurohistochemical (acetylcholinesterase, lactate dehydrogenase, succinate dehydrogenase, nitric oxide synthase, etc.) (8, 12, 16, 17). Nevertheless, AChE histochemistry remains the most suitable method in diagnosing HD, as it is simple, fast, and reliable (8). Our study confirms the reliability of this method in diagnosing HD in infants.

There are several methods for rectal biopsy. Every method has its advantages and disadvantages. When Dobbins and Bill (1965) introduced rectal suction biopsy procedure, which was improved by Noblett (1969), it became the most widely used diagnostic procedure for children with bowel motility disorders. This procedure does not require anesthetics and can be performed in the ward or in the outpatient clinic (18). The disadvantages of this procedure are as follows: it is performed blindly, and

the biopsy site and thickness of the specimen may vary; feces and mucus may influence the thickness of the specimen; the thickness of the specimen may be insufficient because of edematous swollen mucosal layers following x-ray examination or a low suction level. The procedure may result in bleeding, perforation, or sepsis (3, 4, 19, 20). To minimize the risk of complications and to avoid the disadvantages usually caused by the Noblett forceps, various modifications of this instrument have been introduced (6, 19, 20). In our study, rectal biopsy specimens were obtained with rectoscopic forceps during rectoscopy procedure and with scissors through the rectal speculum for older children. Rectoscopy and biopsy were performed under anesthesia. This might be viewed as a disadvantage. However, during the rectoscopy procedure, we could directly inspect the mucous membrane of the rectum in the place of biopsy and the extent of bleeding after the biopsy, and we could stop the bleeding when needed. Other authors also suggest taking biopsy specimens with endoscopic forceps, scissors, or scalpel (5, 18).

Since the year 1964, AChE staining method introduced by Karnovsky and Roots has undergone some modifications. In our study, the modification by El-Badawi and Schenk was chosen, because Harry's hematoxylin stains surrounding tissues and allows better visualization of the relations between these tissues and nervous structures (9). Due to complex technology of preparation and lack of pathologists' experience, AChE histochemistry may be used only in specialized pathology centers (3, 7, 8). However, modern commercial diagnostic sets, using modified lyophilized media, will no doubt increase the number of laboratories applying AChE histochemistry for diagnosing HD (3).

Various studies have indicated that insufficient biopsy specimens, which do not contain submucosa, account for about 34% of all cases (18, 20). In our study, all biopsy specimens included mucosa

and submucosa. Many studies report that the diagnosis of HD may be determined with a 99%–100% accuracy by AChE reaction in the lamina propria and tunica muscularis mucosae alone (6, 15). However, Kobayaschi and et al. state that such a reaction can sometimes be found in normal bowel as well as in IND. Therefore, diagnostic rectal biopsies have to include sufficient submucosa to show the submucosal plexuses and ganglia (10, 21). Our data support this opinion, especially in the case of newborns. However, our study reveals only tendencies, and this topic remains debatable and requires more detailed clinical trials.

Intestinal neuronal dysplasia type B is a controversial condition (8, 22). The majority of authors acknowledge the following diagnostic criteria: increased numbers of nerve cells per ganglion, large ganglion size, large size of a nerve cell, and increased AChE activity in the lamina propria and around submucosal blood vessels (22). The diagnosis should be confirmed according to several diagnostic criteria, though it remains unclear if IND is a separate congenital entity or acquired condition due to chronic constipation (8). In our study, IND was diagnosed by increased AChE activity in the lamina propria and around submucosal blood vessels in the ganglionated bowel.

Conclusions

The analysis of acetylcholinesterase activity in rectal biopsy specimens is a reliable method for diagnosing Hirschsprung's disease, especially in infancy. This test is less valuable in neonates. If test results are negative, infants should be observed, and if symptoms persist, the biopsy should be repeated at the age of 3 months. Rectal biopsy specimens in neonates should include mucosa and submucosa.

Statement of Conflict of Interest

The authors state no conflict of interest.

Acetilcholinesterazės histocheminio tyrimo metodo pritaikymo 20-ties metų patirtis diagnozuojant naujagimių ir kūdikių Hiršprungo ligą

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Raktažodžiai: acetilcholinesterazė, tiesiosios žarnos biopsija, Hiršprungo liga, naujagimiai, kūdikiai.

Santrauka. Žarnos gleivinės ir pogleivio biopsinių preparatų dažymas acetilcholinesterazės (AChE) histocheminiu būdu yra patikimiausias Hiršprungo ligos diagnostikos metodas.

Tyrimo tikslas. Įvertinti acetilcholinesterazės histocheminės reakcijos aktyvumo tyrimo tiesiosios žarnos biopsiniuose preparatuose vertę diagnozuojant Hiršprungo ligą naujagimiams ir kūdikiams

Tiriamųjų kontingentas ir tyrimo metodika. Išanalizuoti 11 naujagimių ir 29 kūdikių, gydytų LSMU Vaikų chirurgijos klinikoje 1991–2010 m., acetilcholinesterazės tyrimo tiesiosios žarnos biopsiniuose prepara-

tuose duomenys. AChE histocheminės reakcijos aktyvumą nervų struktūrose vertinome Karnovskio-Ruts metodu (modifikuotas A. El-Badawi ir E. A. Schenk).

Rezultatai. Dviem naujagimiams diagnozuota Hiršsprungo liga. Devyniems diagnozė buvo paneigta, tačiau trims iš jų progresavo ligos klinikiniai simptomai ir ligos diagnozė patvirtinta tik atlikus pakartotinę biopsiją kūdikiams. Naujagimių tyrimo jautrumas buvo 40,0 proc., specifškumas – 100 proc. 21 kūdikiui diagnozuota Hiršsprungo liga. Visi šie kūdikiiai buvo operuoti. Hiršsprungo ligos diagnozė pasitvirtino 20, vienam diagnozuota B tipo žarnų neurodisplazija. Biopsinio tyrimo rezultatą įvertinome kaip klaidingai teigiamą. Aštuoniems kūdikiams Hiršsprungo ligos diagnozė buvo paneigta. Kūdikių tyrimo jautrumas buvo 100 proc., specifškumas – 88,8 proc.

Išvados. Acetilcholinesterazės aktyvumo tyrimas tiesiosios žarnos gleivinės ir pogleivio biopsiniuose preparatuose yra patikimas Hiršsprungo ligos diagnostikos metodas kūdikiams. Tiriant naujagimius, šio tyrimo vertė yra mažesnė, todėl esant neigiamiems biopsijos rezultatams kūdikiiai turi būti stebimi. Jeigu simptomai išliktų, biopsija turi būti kartojama 3 mėn. amžiaus kūdikiui. Tiesiosios žarnos biopsija, tiriant naujagimius, turi apimti gleivinės ir pogleivio sluoksnius.

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