

The evaluation of rectal mucosal punch biopsy in the diagnosis of Hirschsprung's disease: a 30-year experience of 954 patients

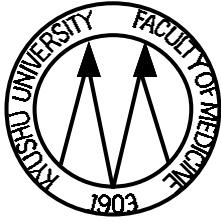
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Prem Puri

Editor-in-Chief, Pediatric Surgery International

Dear Prof. Prem Puri

We are pleased to submit our manuscript entitled, “**The evaluation of rectal mucosal punch biopsy in the diagnosis of Hirschsprung’s disease: A thirty-year experience of 954 patients**” for consideration for publication as an original article in *Pediatric Surgery International*.

In this study, we evaluated the safety of the punch biopsy procedure including “K-PUNCH” biopsy and open punch biopsy as a rectal mucosal biopsy in the diagnosis of Hirschsprung’s disease and its allied disorder, intestinal neuronal dysplasia type B. Nine hundred and fifty-four patients underwent punch biopsy at our institution and our branch hospitals since April 1986. A high successful rate of biopsy with a low incidence of complications (such as a bleeding) was obtained. Our technique is therefore considered to be a safe and feasible method for diagnosing Hirschsprung’s disease.

We believe that the results of our retrospective study will provide important information that would be valuable to your readers.

The manuscript contents are original and have not been published or accepted elsewhere, either in whole or in part, in any form other than an abstract. This manuscript is not being considered for publication in another journal and it will not be submitted elsewhere if it is accepted by *Pediatric Surgery International*, nor will it be submitted elsewhere before a decision has been reached by the Editorial Board.

KY and YK participated in the design, data analysis, research, and writing of the manuscript; YY, SO, TJ, TI, YT, GE, JAM, TM and TI provided helpful advices; TT reviewed the manuscript and supervised the whole study process. In addition, they also declare no conflict of interests in association with this study.

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We are looking forward to hearing from you.

Yours sincerely,

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The evaluation of rectal mucosal punch biopsy in the diagnosis of Hirschsprung's disease: A thirty-year experience of 954 patients

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Abstract

Purpose: For 30 years, we have consecutively performed rectal mucosal punch biopsy to diagnose Hirschsprung's disease. The aim of this study was to evaluate the safety of our technique.

Methods: Patients with suspected Hirschsprung's disease who underwent punch biopsy, including our original "K-PUNCH" method using an S-moid forceps and non-specific blood-collecting tube at our department and branch hospital between April 1986 and March 2016 were included in the present study. Our punch biopsy technique is characterized by excellent visibility and a direct grasping sensation. The backgrounds and complications of the patients were retrospectively investigated.

Results: During this period, 954 patients (median age, 4 months; range, 1 day to 73 years) underwent punch biopsy. Although there were no cases of severe complications (i.e. rectal perforation, infection or full-thickness biopsy), one (0.1%) of the 954 cases in the early period showed liver dysfunction and required transfusion due to bleeding. In addition, inappropriate specimens were obtained in 37 patients (3.9%).

Conclusion: Punch biopsy including the "K-PUNCH" method is considered safe and feasible and is associated with a low rate of complications and inappropriate specimen harvesting among patients of all ages. Comorbidities, including the potential for hemorrhage, should always be considered.

Keywords:

Hirschsprung's disease; diagnosis; punch biopsy; rectal mucosal biopsy; acetylcholinesterase staining.

Abbreviations:

HD, Hirschsprung's disease; RMB, rectal mucosal biopsy, AChE, acetylcholinesterase; IND-B, intestinal neuronal dysplasia type B.

Introduction:

Hirschsprung's disease (HD) is a well-established congenital motility disorder, characterized by functional bowel obstruction, which occurs due to the absence of enteric ganglion cells [1]. A histopathological examination is therefore mandatory for the diagnosis of HD. Open wedge full-thickness biopsy with Auerbach's myenteric plexus was first described by Swenson et al. [2]; however, this method, which requires general anesthesia, is invasive and is associated with complications including complicated bleeding, infection, and/or scarring. In 1960, Gherardi et al. [3] demonstrated that the submucosal ganglion cells in the rectum presented at the same level as the myenteric plexus, and that full-thickness biopsy was no longer required. With regard to rectal mucosal biopsy (RMB) methods, suction biopsy was first described by Dobbins et al. [4] and refined by Noblett [5], while punch biopsy was first described by Shandling [6]. Although there have been several reports on the complications associated with this procedure [7-20], various refinements have made rectal suction biopsy a safe technique, and the evolution of this procedure has established rectal suction biopsy as the current gold standard technique for RMB [15-17, 21-23]. From 1977, we also began to perform rectal suction biopsy using the Truelove Salt Biopsy Instrument (Van Bros Co, London, England); since then, more than 500 rectal suction biopsies have been performed in our department. However, in 1986, we began to perform punch biopsy using the so-called "K-PUNCH" method and open punch biopsy due to a number of problems with suction biopsy [24], and we have consecutively performed punch biopsy so far [24-26].

From the 1990s, the indication for RMB was expanded to include the diagnosis of intestinal neuronal dysplasia type B (IND-B) in the allied disorders of Hirschsprung's disease [27]. IND-B is known to occur in adulthood as well as childhood [28]; thus, safe biopsy techniques are necessary to accurately diagnose IND-B in pediatric and adult patients with chronic constipation,

The present study describes our 30-year experience of punch biopsy for the diagnosis of HD and IND-B, which was associated with a very low incidence of complications. The present study also describes the procedure in detail and focuses on the diagnoses and complications of the techniques or specimens.

Methods:

Patients

Patients in whom HD was suspected, and who underwent RMB in our department or branch hospitals between April 1986 and March 2016 were included in the present study. Our indications for RMB included the presence of a symptom that was indicative of intestinal obstruction (such as a bilious vomiting, chronic constipation or abdominal distension) and the presence of abnormal dilatation of the intestine on a radiography (including the obvious or suspected presence of a "caliber change" in the transit zone of HD in contrast enema). Manometry was not performed as a routine examination. The patients' backgrounds, the incidence of complications (such as bleeding requiring transfusion, perforation,

infection, and full-thickness biopsy) and the histopathological outcomes were retrospectively investigated after dividing the patients into 4 age groups: neonates (<30 days); infants (1-11 months); children (1-17 years); adults (>18 years).

Punch biopsy

We usually perform punch biopsy without general anesthesia in the outpatient department. Prior to biopsy, rectal preparation is usually performed to obtain an appropriate sample. During the biopsy procedure, sedative agents are only required for anxious and/or uncooperative older children.

We performed two types of punch biopsy according to the patient's age and and/or the size of their rectum; The first type is punch biopsy using the so-called "K-PUNCH" procedure, which was reported in detail by Hirose et al. in 1993 [24]. Briefly, 1) an S-moid forceps (Nishihata) (No. 15602; NAGASHIMA MEDICAL INSTRUMENTS CO., LTD., Tokyo, Japan) with a cup size of 3mm, and a non-specific blood-collecting tube with a 6-mm hole drilled at the top of the tube and a point marked at 3cm from the drilled hole to indicate the distance from the dental line in a timely manner during biopsy are prepared prior to the biopsy procedure (**Fig. 1a**). 2) Patients are fixed in the lithotomy position by an assistant and the operator's little finger is inserted into the rectum to exclude anal stenosis. 3) A non-specific blood-collecting tube with a 6-mm hole drilled into the top of the tube is gently inserted to the rectum (**Fig. 1b,c**). 4) Appropriate lateral pressure is applied in order to fix the side aperture securely

against the mucosal surface. The protrusion of the rectal mucosa into the internal cavity is then clearly observed with the full view of the operator under a pen light (**Fig. 1d**). With regard to the tube, a non-specific blood-collecting tube is generally used; however, a 1.5-ml micro tube is occasionally used for neonates and a 15-ml centrifuge tube is occasionally used for older children depending on the size of rectum (**Fig. 1e**); The other technique was open biopsy using an S-moid forceps (Nishihata) through a proctoscope under the operator's direct vision. This procedure was indicated for older children or adults, whose rectum was too dilated to obtain the adequate lateral pressure during K-PUNCH method. Following the identification of the appropriate biopsy site by two above-mentioned methods, the mucosa was completely grasped with the above-mentioned laryngeal S-moid forceps and the specimens are pulled off with the resistance disappearing at up to 1-2 seconds (**Fig. 1f**). The specimens were immediately preserved in liquid nitrogen for acetylcholinesterase (AChE) staining. If the specimen could not be obtained with the above-mentioned resistance, the grasped specimen was released in order to avoid massive bleeding and unnecessary deep-layer biopsy. In contrast, when the specimen could be obtained without any resistance, this indicated that the harvested specimen was too shallow to be adequate. This principle was commonly applied, regardless of the age of the patients. Appropriate resistance modulated by the operator while grasping the tissue is most important for obtaining an appropriate specimen. Two or three additional biopsies were immediately performed at other portions of the rectum wall while the concurrent turning of the biopsy tube contributed to the compressive hemostasis of biopsy site in

K-PUNCH biopsy or after direct compression with sterilized gauze in open biopsy. Finally, complete hemostasis of slight transient rectal bleeding was achieved at the end of the biopsy procedure by packing with gauze for 30 minutes. With regard to the criteria that determined the selection of the punch biopsy procedure, “K-PUNCH” biopsy was routinely performed in all the neonates and infants; open biopsy was routinely performed in adults; in children, the operator decided whether “K-PUNCH” biopsy or open biopsy was preferable in accordance with the patient’s age and/or the size of their rectum.

With regard to the appropriate biopsy site, biopsies were carried out at an appropriate distance above the dental line according to the patient’s age (1.5-2.0 cm, <1 month-old; 2.0-2.5cm, < 1 year-old; 2.5-3.0 cm, < 3 years-old; 3.0-3.5cm, >4 years-old) in order to avoid confusion with physiological zones of hypoganglionosis [29-31].

AChE staining

An enzyme-histochemical technique, AChE staining, was performed according to the manufacturer’s instructions, as previously described [24-26]. The samples were compounded with optimal cutting temperature (OCT), sliced at a thickness of 10 µm using a cryostat (SAKURA Precision Instrument Co, Tokyo, Japan) and thaw-mounted onto glass slides. For the staining of AChE activity, we used a modification of the method of Karnovsky and Roots, with rubeanic acid used as an amplifier [32-34]. The following definitions were applied in present study. The cases lacking ganglion cells in the

submucosal layer and in which thick AChE-positive fibers were present were defined as HD [35]. With regard to the diagnostic criteria of IND-B, the criteria have since been revised by the Meier-Ruge group [36, 37]. The present diagnostic criteria are: (1) more than 20% of submucosal ganglia containing >9 ganglion cells (with at least 25 ganglia evaluated) and (2) older than 1 year of age [37, 38]. The cases in which normal ganglia were observed with a normal number of ganglion cells in the submucosal layer, with or without thin AChE-positive fibers were defined as normal. The cases in which insufficient tissue was harvested due to the absence of the submucosal layer or malposition because a squamous epithelium covered the area near the dental line were defined as, “inappropriate” specimens [29, 30]. Appropriate specimens that met the criteria for either HD, IND-B or normal were categorized as “undetermined”. Despite the use of a special cool delivery service, these specimens melted during transportation from the branch hospital. They were therefore considered to be in a “bad condition”.

Ethical Statement

This retrospective study was performed according to the Ethical Guidelines for Clinical Research, published by the Ministry of Health, Labour and Welfare, Japan on July 30, 2003 (revised in 2008). The study complied with the 1964 Declaration of Helsinki (revised in 2008). Written informed consent was obtained from each of the patients and/or their parents or guardians prior to the performance of the biopsy.

Results:

During the study period, a total of 954 patients underwent punch biopsy. The background information of the patients is shown in **Table 1**. The median age of all 954 patients (male, n=557) was 4 months (range, 1 day - 73 years). The diagnoses of the 954 patients were as follows: HD, n=289 (30.3%) (male, n=217; median age, 3 months; range, 1 day - 73 years); IND-B, n=15 (1.6%) (male, n=10; median age, 3 years; range, 1 year - 7 years); normal patients, n=569 (59.7%) (male, n=281; median age, 6 months of median; range, 1 day - 69 years); “undetermined”, n=34 (3.6%) (male, n=19; median age, 1 year; range, 1 month - 47 years); and “inappropriate”, n=37 (3.9%) (male, n=23; median age, 3 years; range, 20 days - 62 years). The detailed diagnoses of the patients in each group are shown in **Table 2**.

Among 290 neonates, 129 (44.5%) were diagnosed with HD and 151 (52.1%) were diagnosed as normal.

Among 364 infants, 123 (33.8%) were diagnosed with HD and 218 (59.9%) were diagnosed as normal.

Among 262 children, 33 (12.6%) were diagnosed with HD, 15 (5.7%) were diagnosed with IND-B and 172 (65.6%) were diagnosed as normal. Among 38 adults, 4 (10.5%) were diagnosed with HD and 28 (73.7%) were diagnosed as normal. Seven (2.4%) and 3 (1.0%) of the 290 neonates; 10 (2.7%) and 8 (2.2%) of the 364 infants; 15 (5.7%) and 23 (8.8%) of the 262 children; and 2 (5.3%) and 3 (7.9%) of the 38 adults were classified as “undetermined” and “inappropriate”, respectively.

With regard to severe complications, bleeding requiring transfusion only occurred in one

patient who had coagulopathy due to severe liver dysfunction in 1991, which is the early period of this study; whereas, rectal perforation, infection and full thickness biopsy did not occur in the study period (**Table 3**).

Discussion:

HD was definitively diagnosed based on the pathological confirmed absence of submucosal ganglion cells and the presence of hypertrophic nerves. Thus, RMB—which requires an appropriate tissue sample—is mandatory for the definitive diagnosis of HD. Suction biopsy and punch biopsy are currently available as RMB methods, with suction biopsy clearly the gold standard due to its very low rate of complications [39]. On the other hand, we have consecutively performed punch biopsy since 1986 when Hirose et al. reported its safety [24]. At least 15 operators performed punch biopsy during this study period and the low complication rates have thus far been maintained. To the best of our knowledge, other than our own reports [24-26], only three reports on punch biopsy methods have been published since the initial report of Shandling [6]; however, two of the 3 reports described punch biopsy combined with endoscopy [40, 41]. Thus, the study by Alizai et al., which noted that 102 rectal mucosal punch biopsies with no major complications such as bleeding requiring blood transfusion, intestinal perforation and sepsis and 4% of inappropriate sample rates, is the only other detailed report in the literature [14]. Thus, this is the first study to elucidate the safety and efficacy of punch biopsy in a large population over a

30-year period.

The most important factor in successfully performing a RMB is obtaining a biopsy site with an adequate and consistent level [42]. Thirty-seven (3.9%) of 954 patients were deemed to be “inappropriate” in the present study, which is acceptable low rate as well as the report described by Alizai et al [14]. This low rate may be due to 3 special features of punch biopsy using an S-moid forceps: the strict confirmation of the distance between the dental line and the biopsy site (which avoids biopsy from the anal squamous epithelium); the direct grasping sensation transmitted through the S-moid forceps; the 3-mm cup size of the S-moid forceps (which helped ensure a safe biopsy by avoiding the retrieval of an unnecessarily large sample). Using this size was thought to be acceptable because the international Working Group of the 2009 World Congress of Gastroenterology stated that the diagnosis of HD requires a biopsy specimen of at least 3 mm in diameter [43]. These great advantages, which were achieved through direct visualization with a full view and the ability to perform sensitive handling maneuvers using an S-moid forceps, also clearly contributed to the low rate of complication, even in neonates and infants younger than 1 year of age.

On the other hand, the children and adults group had a relatively higher “inappropriate” rate. A possible reason for this is the difficulty in obtaining an accurate biopsy specimen due to the age of the patient because morphological changes, such as an increase in the thickness of the rectal wall, mucosal edema and fibrous tissue, may occur in older patients who receive chronic enemas and who subsequently

develop edema of the mucosal layer [28, 44]. Furthermore, the longer distance of the normal hypoganglionic or aganglionic segment from the dental line in older children and adults—which gradually increased from 1.0 cm in the neonatal period to approximately 3.5 cm in older children and adults—may have had an impact on the difficulty of biopsy in children and adults groups [31]. Thus, RMB is considered to be difficult in older children and adults, and the proposal of RMB in patients within this age range remains controversial and challenging problem at present [45].

Some reports, however, have described children and adult patients including 67-year-old women with the chronic constipation being diagnosed with HD [46-49]. Moreover, acute intestinal obstruction, perforation and volvulus sometimes overlap with HD in children and adults [50-52]. Actually in the present study, 33 children and 4 adults were diagnosed with HD. IND-B should also be taken in account because it is a possibility in children who suffer from chronic constipation [28]. These previous reports and our results indicate that RMB in this age range has an important role for the undelayed diagnosis of HD and IND-B. To overcome this challenging problem, the further refinement of devices and techniques will be required; this may involve the careful use of a forceps with larger tips or the use of a biopsy tube with a larger side hole, but only for when children and adults undergo punch biopsy in accordance with the patient's status.

In conclusion, the present study revealed the safety, efficacy and versatility of punch biopsy for patients of various ages, ranging from neonates to older adults (range, 1 day to 73 years). This result may

indicate the feasibility of punch biopsy for rectal mucosal biopsy. We should keep in mind that “inappropriate” specimens will occasionally be harvested, especially in children and adults and that comorbidities, including hemorrhage, may occur in complicated patients.

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Compliance with Ethical Standards:

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study

formal consent is not required.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure legend

Fig1. The detailed technique of “K-PUNCH” biopsy. An S-moid forceps (Nishihata) and a handmade rectal biopsy tube with a 3-mm hole drilled at the top of tube. The tube is marked at 3 cm from the drilled hole (a). A schematic drawing representing the insertion of the tube into the rectum from the lateral view. The insertion of the tube causes the rectal mucosa to protrude. The rectal mucosa is then grasped by the forceps (indicated by the asterisk). The white arrow indicates the anal verge; the black arrow indicates the dental line (b). The external appearance during the “K-PUNCH” biopsy procedure. The tube is fixed by the left hand to stabilize the operator’s view (c). The internal view during the “K-PUNCH” biopsy procedure. A full, clear view of the operative field is achieved. The black arrow indicates the protruding rectal mucosa (d). A specimen of appropriate size was harvested. The black arrow indicates the specimen (e). Three types of tube are ordinarily used in accordance with patient’s side. The tube in the upper position is a 15-ml centrifuge tube, the tube in the middle is a non-specific vessel tube, and that in the lower position is 1.5-ml micro tube. The diameters of all of the tubes are written on the lateral part of the tube in order to measure the distance from the dental line during biopsy (f).

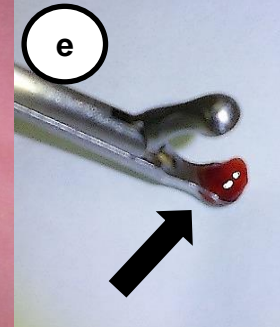
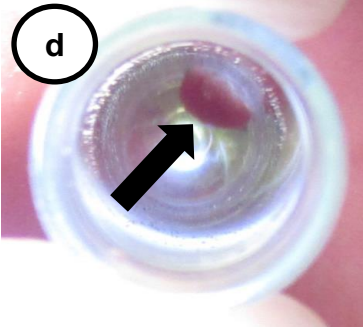
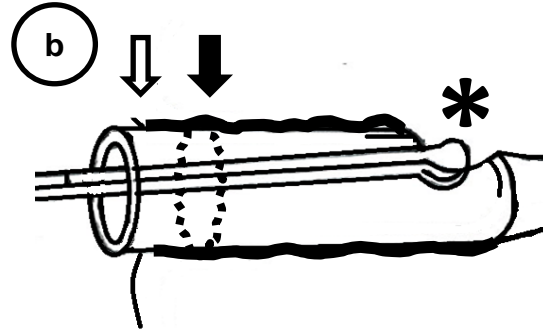
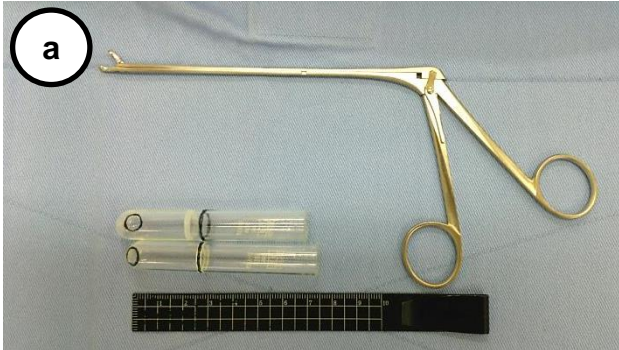


Table 1. The background characteristics of the 954 patients who underwent “K-PUNCH”

			Age		Sex
			Median	(Range)	M : F
Total	954	(100%)	4 months	(1 day - 73 years)	557 : 397
Diagnosis					
HD	289	(30.3%)	3 months	(1 day - 73 years)	217 : 72
IND-B	15	(1.6%)	3 years	(1 year - 7 years)	10 : 5
Normal	569	(59.7%)	6 months	(1 day - 69 years)	281 : 288
Undetermined	34	(3.6%)	1 year	(1 month - 47 years)	19 : 15
Inappropriate	37	(3.9%)	3 years	(20 days - 62 years)	23 : 14
Bad condition	10	(1.0%)	2 years	(3 months - 11 years)	7 : 3

Abbreviations: HD, Hirschsprung’s disease; IND-B, intestinal neuronal dysplasia type B; M, male; F, female

Table 2. The diagnoses of the 954 patients who underwent punch biopsy

Group	Neonates (< 30 d)	Infants (1-11 m)	Children (1-17 y)	Adults (≥ 18 y)	Total
n (% in each group)	290 (100)	364 (100)	262 (100)	38 (100)	954 (100)
HD	129 (44.5)	123 (33.8)	33 (12.6)	4 (10.5)	289 (30.1)
IND-B	0 (0)	0 (0)	15 (5.7)	0 (0)	15 (1.6)
Normal	151 (52.1)	218 (59.9)	172 (65.6)	28 (73.7)	569 (59.6)
Undetermined	7 (2.4)	10 (2.7)	15 (5.7)	2 (5.3)	34 (3.6)
Inappropriate	3 (1.0)	8 (2.2)	23 (8.8)	3 (7.9)	37 (3.9)
Bad condition	0 (0)	5 (1.4)	4 (1.5)	1 (2.6)	10 (1.0)

Abbreviations: HD, Hirschsprung's disease; IND-B, intestinal neuronal dysplasia type B; d,

days; m, months; y, years.

Table 3. The incidence of severe complications

Severe complications		
Bleeding requiring transfusion	1	(0.1%)
Rectal perforation	0	(0%)
Infection	0	(0%)
Full-thickness biopsy	0	(0%)
Total	954	(100%)
