Hirschsprung's disease: early diagnosis and long-term outcomes

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CHAPTER 2

Infant's age influences the accuracy of rectal suction biopsies for diagnosing of Hirschsprung's disease

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SUMMARY

Background

Hirschsprung's disease (HD) is a rare birth defect of the distal colon. Analysis of rectal suction biopsy (RSB) is considered to be the most reliable method for its diagnosis in infants. However, the diagnostic accuracy of RSB analysis could be affected by the patient's age, possibly because of rapid development of the enteric nervous system in the first weeks after birth. Because there is a trend toward testing for HD at early ages, we aimed to determine whether the diagnostic accuracy of RSB analysis is associated with the patient's age.

Methods

We performed a retrospective analysis of all patients from whom one or more RSBs were analyzed from 1975 through 2011 (529 RSBs from 441 patients). Outcomes of RSB analyses were categorized as positive, inconclusive, or negative for HD. Primary diagnoses, based only on RSB, were compared with final diagnoses made after at least one year of clinical follow-up. Age at time of RSB analysis was corrected for the gestational age. By using these criteria, we determined the diagnostic accuracy of RSB analysis for different age groups.

Results

RSB analysis identified HD in patients with sensitivity values of 46% (patients –45 to 7 days old), 47% (8–22 days old), and 62% (23–39 days old) (corrected for gestational age). The average sensitivity with which RSB analysis identified HD in patients older than 39 days was 88%. RSB identified HD in patients younger than 39 days old with significantly lower sensitivity than in older patients (50% vs 88%, $P < .001$). The specificity with which RSB identified infants without HD was not affected by age (average 95%). Of all RSBs, 11% were inconclusive for the diagnosis of HD.

Conclusions

RSB analysis identifies HD in patients younger than 39 days old with only 50% sensitivity. Moreover, RSBs obtained from younger patients often lead to inconclusive outcomes and require additional biopsies. We propose that for infants suspected of HD at these ages, a noninvasive technique, such as anorectal manometry, should be used for a primary diagnosis. RSB should thereafter be used to confirm the diagnosis when the infant is older than 39 days.

INTRODUCTION

Hirschsprung's disease (HD) is a relatively rare birth defect of the distal colon. It is characterized by aganglionosis, *i.e.* the absence of ganglion cells in the enteric nervous system. At the same time, these patients also miss the rectoanal inhibitory reflex due to aganglionosis in the internal sphincter complex.[1] The incidence of HD is estimated to be approximately 1 in 5000 live births.[2] Children with constipation complaints, who are suspected of having HD, are seen far more often.

While clinical presentation can only suggest HD, the final diagnosis must be confirmed by the outcomes of rectal suction biopsy (RSB), contrast enema, and/or anorectal manometry. All three tests have been shown to have similar sensitivity and specificity, with the RSB being the most reliable.[3] Anorectal manometry and contrast enema were previously described as difficult to interpret in the neonatal period.[4–8] Additionally, recently, Bagdzevicius and colleagues[9] reported that RSB too is not an entirely satisfactory method for diagnosing HD in neonates since its outcome may be affected by the patient's age. A possible explanation for these diagnostic obstacles is the dynamic increase of hypertrophic nerve fibers during the first weeks after birth.[10] These nerve fibers are of an extrinsic origin. In HD patients they become hypertrophic and proliferate due to a failure to connect with the intrinsic, enteric nervous system. Both the intrinsic absence of ganglion cells and hypertrophy with proliferation of extrinsic nerve fibers can be used in diagnosing HD. Currently, there is a trend towards diagnosing HD increasingly early, with the majority of patients already tested during the neonatal period.[11] While this trend is thought to decrease the risk of life threatening Hirschsprung's-associated enterocolitis,[12] it may at the same time introduce the risk of misdiagnosing HD.

Furthermore, we also observed that the diagnosis of HD is often very troublesome in newborns. On the basis of this clinical experience, combined with the knowledge from scientific literature, we hypothesized that the outcome of the RSB could be dependent on the age of the patient. The aim of this study was, therefore, to determine at which age RSB provides an accurate diagnosis.

METHODS

Data collection

A retrospective analysis was conducted on suspected HD patients who had undergone one or more rectal suction biopsies (RSBs) between 1975 and 2011 at University Medical Center Groningen. Indications for performing a RSB were: delayed meconium passage, distended abdomen, difficult spontaneous defecation, and/or signs of Hirschsprung's-

associated enterocolitis. The age at which the RSBs were obtained was corrected for the infant's gestational age. A full term age of 40 weeks was considered normal. During the study period five pathologists with several years' experience in academic gastrointestinal pathology and who were familiar with the diagnosis of HD examined the RSBs. The pathologists succeeded each other over the years, wherein a maximum of two pathologists were responsible for the analysis of RSBs at any given time. RSBs were excluded from the main statistical analysis if the pathology report stated that the RSB was inappropriate for diagnosing HD. In these patients the RSB contained insufficient submucosa or anal epithelium. The RSBs that yielded sufficient tissue were either classified as positive for HD (no ganglion cells and/or increased nerve fiber proliferation), inconclusive for HD (no ganglion cells and little or no nerve fiber proliferation), or negative for HD (ganglion cells and little or no increased nerve fiber proliferation). In order to find possible factors that contributed to the diagnostic accuracy of the RSB outcomes we analyzed the RSBs that had been rejected by the pathologists due to insufficient tissue.

RSB and staining procedures

Prior to obtaining a RSB, the patient was given a cleansing enema. Subsequently, rectal tissue consisting of mucosal and submucosal material was extracted using the rectal suction biopsy tube, specially designed for use in HD.[13] The RSB procedure was performed without sedation or anesthesia, unless it was required for other another procedure. In all patients the RSB procedure consisted of extracting specimens at three levels between 2 and 10 cm, varying between physicians, above the anal verge (*e.g.* 3, 4.5, and 6 cm). The specimens were frozen and examined at, on average, six levels at 200 µm intervals. At each interval a 10 µm thick section was stained by hematoxylin and eosin (H&E), reduced nicotinamide adenine dinucleotide (NADH) enzyme histochemistry, and acetylcholinesterase (AChE) enzyme histochemistry. The presence of ganglion cells excluded the diagnosis Hirschsprung's disease. Hypertrophy or hyperplasia of extrinsic cholinergic nerve fibers in the submucosa, muscularis mucosa, and/or mucosa together with the absence of ganglion cells, was regarded as compatible with Hirschsprung's disease. NADH histochemistry (Roche, Switzerland) was performed in combination with tetrazolium reductase (Sigma, United Kingdom), as was reported elsewhere.[14] The AChE histochemistry staining technique was applied according to a modified version of the method described by Karnovsky and Roots.[15]

Analysis of the RSB data

We compared the diagnosis based on the primary RSB to the final diagnosis made after at least one year of clinical follow-up. The final diagnosis was based on the clinical

condition of the patient, additional RSB results, anorectal manometry and/or resected colon pathology. An exclusion factor for HD was the presence of a functioning rectoanal inhibitory reflex as determined by anorectal manometry. The RSB outcomes were classified as true positive, false positive, true negative, or false negative on the basis of the final diagnosis.

In order to analyze the RSB outcomes at different ages, we divided the patients into ten groups on the basis of their age percentiles: -45 to 7, 8 to 22, 23 to 39, 40 to 53, 54 to 78, 79 to 108, 109 to 160, 161 to 246, 247 to 335, and 336 to 6390 days of age (corrected for gestational age). The sensitivity and specificity of the RSB were calculated separately for each age group. We also determined the probability of a correct HD diagnosis based on the age at which the RSBs were obtained.

Statistical analysis

Data were analyzed with SPSS 20.0 for Windows (IBM SPSS Statistics, IBM Corporation, Armonk, NY). The statistical tests that were used were limited to Pearson's chi-square test, Student's *t*-test, and Mann-Whitney *U*-test, which were all used when the requirements were met. Sensitivity and specificity of the test were calculated using the classification mentioned above, as appropriate. Predictive values were calculated with the same classifications, as is conventional. The probability was defined by the number of true positive RSB outcomes. The relationship between age and the probability of a correct diagnosis was evaluated by spline regression analysis using Stata 11 (StataCorp, College Station, TX). Two-sided *P* values of less than .050 were considered statistically significant.

Table 1
Patient characteristics

Patients who underwent a RSB (n)	441
One RSB needed for accurate diagnosis (%)	83%
Multiple RSBs needed for accurate diagnosis (%)	17%
Patients ultimately diagnosed with HD (n)	190
Total number of RSBs obtained (n)	559
Included (sufficient) RSBs (n)	529
Excluded (insufficient) RSBs (n)	30
Median age at time of RSB (days)*	78 (-45 – 6390)

HD = Hirschsprung's disease, RSB = rectal suction biopsy
* Median (minimum – maximum)

RESULTS

Patient characteristics

Out of a total of 559 RSBs obtained between 1975 and 2011, we excluded 5.4% (n = 30) because insufficient tissue had been extracted (Table 1). Hence, we included 529 RSB outcomes obtained, from 441 patients in our analyses. Patients' ages at the time of the biopsies ranged from -45 days in the youngest to 6390 days, *i.e.* 17 years and 6 months, in the oldest. The median age was 78 days. Based on clinical follow-up, additional RSBs, anorectal manometry, or pathology of the resected colon, we diagnosed 43% of the patients (n = 190) with HD after at least one year of follow-up. In 57% of the patients (n = 251) we excluded HD. Out of the 441 patients who had undergone RSBs, 17% (n = 74) required one to three additional RSBs to arrive at an accurate diagnosis. The highest number of additional RSBs that were necessary to either confirm or exclude HD was observed in the group of 8 to 22-day-olds, and the lowest in the group of 161 to 246-day-olds (Figure 1, $P < .001$).

Patients' age determines the sufficiency of rectal suction biopsy material

Irrespective of patients' ages, 5.4% (n = 30) of the RSBs had to be excluded because insufficient tissue had been extracted. In order to identify the possible risk factors of RSBs that provided insufficient tissue, we compared the excluded RSB data with the

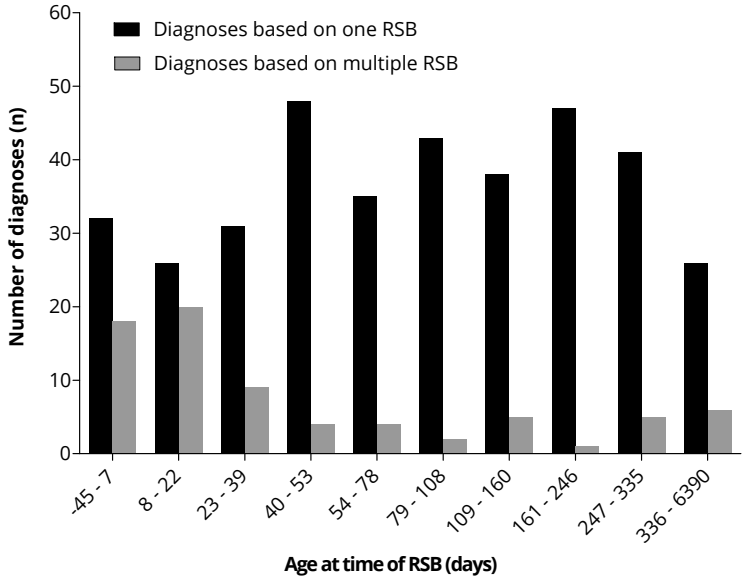

Figure 1
The number of single (black) or additional (white) RSBs needed to confirm or exclude the diagnosis of HD at different age groups ($P < .001$).

Table 2
Accuracy of RSB at different ages

Ages of patients at time of RSB (days)	No. of RSBs per group (n)	RSBs obtained in HD patients					RSBs obtained in non-HD patients				
		True positive	Inconclusive	False negative	Total	Sensitivity (%)	True negative	Inconclusive	False positive	Total	Specificity (%)
-45 - 7	55	17	12	8	37	**46**	18	0	0	18	**100**
8 - 22	54	21	15	9	45	**47**	8	1	0	9	**89**
23 - 39	50	18	5	6	29	**62**	20	1	0	21	**95**
40 - 53	54	25	2	1	28	**89**	24	2	0	26	**92**
54 - 78	53	20	3	2	25	**80**	27	1	0	28	**96**
79 - 108	52	22	0	0	22	**100**	29	1	0	30	**97**
109 - 160	53	12	2	1	15	**80**	37	1	0	38	**97**
161 - 246	54	17	0	0	17	**100**	37	0	0	37	**100**
247 - 335	52	7	2	0	9	**78**	38	4	1	43	**88**
336 - 6390	52	33	5	0	38	**88**	13	1	0	14	**93**
	529	192	46	27	265	**72**	251	12	1	264	**95**

HD = Hirschsprung's disease, RSB = rectal suction biopsy

remaining RSB data that did consist of sufficient tissue. We found a statistically significant difference (P = .033) in age of obtaining the RSBs between the excluded RSBs and the included RSBs: median 43 days (interquartile range, 13-114) in insufficient RSBs versus 78 days (interquartile range, 30-190) in sufficient RSBs. Moreover, we found that insufficient tissue had been extracted significantly more often in patients without HD than in patients with HD (8.3% versus 3.0%, P = .013).

Accuracy of rectal suction biopsy at different ages

We described the accuracy of the RSB for diagnosing HD correctly in terms of three parameters; sensitivity, specificity, and probability.

The overall sensitivity of the RSB outcomes, irrespective of age, was 72% (Table 2). The sensitivity did not change significantly over the studied period of 36 years (Figure 2, P = .315). In patients aged -45 to 7, 8 to 22, and 33 to 39 days, HD was correctly diagnosed on the basis of RSB outcomes in 46%, 47%, and 62% cases, respectively. In patients older than 39 days, the lowest sensitivity of RSB outcomes was 78% in the group of 247 to 335-day-olds. A sensitivity of 100% was reached in the groups of 79 to 108-day-olds and 161 to 246-day-olds.

Based on these observations we decided to continue our analysis of sensitivity in two age groups: the RSB obtained from patients younger than 39 days and those of patients older than 39 days. Subsequently, we observed that the sensitivity of RSB obtained from patients younger than 39 days was significantly lower than the sensitivity for patients older than 39 days (50% versus 88%, P < .001, Table 3).

We found no significant difference in specificity among the age groups investigated (Table 2). The average specificity of the ten groups combined was 95%.

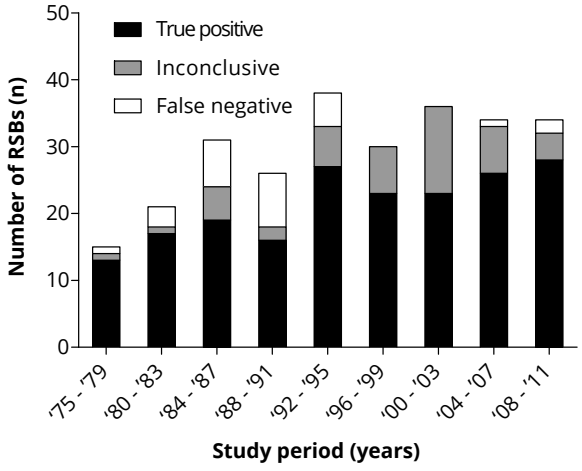

Figure 2
The accuracy of RSBs obtained from HD patients in consecutive study periods.

Because we found that the sensitivity of RSB tended to increase with increasing age, we decided to determine the probability of an accurate HD diagnosis at different ages. The probability of an accurate HD diagnosis was plotted against the age at the time the RSB was obtained (Figure 3). We found a gradual increase in probability up to a maximum value at approximately 125 days (Figure 3A). After the initial increase of probability we found an evidently decreasing trend as patients' ages increased (Figure 3B).

Inconclusive rectal suction biopsy outcomes

Unfortunately, 11.0% of the RSB outcomes were inconclusive (Table 2). The highest number of inconclusive outcomes was obtained from 8 to 22-day-old infants (3.2%). We found the lowest ratio of inconclusive outcomes (0.2%-0.6%) in older infants, *i.e.* 79- to 246-day-olds. If the pathologist had classified a RSB outcome as inconclusive for HD, it had a predictive value of 79% for eventually confirming HD.

Twelve RSB outcomes from eleven different patients were inconclusive for HD and were ultimately diagnosed as non-Hirschsprung (Table 2). Nevertheless, a redo of RSBs was required in seven patients to exclude HD beyond all doubt in these cases. Two patients, in whom the primary HD diagnoses were inconclusive, had complete resolution of defecation complaints after a difficult start. As a consequence, no additional diagnostic tests needed to be administered. The tenth patient underwent two RSBs, on the basis of which he was suspected of having an ultra-short variant of HD. This patient subsequently underwent a lateral sphincterectomy to remedy his persistent constipation complaints.

Table 3
The RSB outcomes for two different age groups

Diagnosis	Outcome	RSBs, ≤ 39 days of age	RSBs, ≥ 40 days of age	*P* value
HD patients	True positive RSBs (n)	56/111 (50%[a])	136/154 (88%[a])	< .001
	Inconclusive RSBs (n)	32/111 (29%)	14/154 (9%)	< .001
	False negative RSBs (n)	23/111 (21%)	4/154 (3%)	< .001
Non-HD patients	True negative RSBs (n)	46/48 (96%[b])	205/216 (95%[b])	NS
	Inconclusive RSBs (n)	2/48 (4%)	10/216 (5%)	NS
	False positive RSBs (n)	0/48 (0%)	1/216 (0%)	NS

HD = Hirschsprung's disease, RSBs = rectal suction biopsies
a Sensitivity
b Specificity

Unfortunately, both the RSB outcomes were inconclusive, and the diagnosis turned out to be a false positive, since the anorectal function tests performed at the age of nine years showed a functioning rectoanal inhibitory reflex. In the eleventh patient for whom the HD diagnosis was inconclusive, the rectoanal inhibitory reflex was found to be absent by anorectal manometry when he was two years old. The outcomes of the RSB and anorectal manometry led to the conclusion to perform a Duhamel reconstruction. Pathological examination following surgery showed the presence of ganglion cells at both the distal and the proximal ends. Although an ultra-short variant of HD in this last patient could not be ruled out completely by the pathological findings, it is more likely that the RSB outcomes were not conclusive enough for making an accurate diagnosis.

False positive rectal suction biopsy outcomes

We had only one false positive RSB outcome. This RSB showed no ganglion cells at 3 and 4.5 cm and hypertrophy with proliferation of nerve fibers at 3, 4.5, and 6 cm. We suspected a shorter variant of HD. Later, this diagnosis had to be corrected. Anorectal function testing at the age of 18 years showed a functioning rectoanal inhibitory reflex. Fortunately, this patient had not undergone any surgery. Instead, he had received conservative treatment in the form of laxatives and daily rectal flushing following the diagnosis of HD based on RSB outcome.

DISCUSSION

In this study we demonstrate for the first time that a patient's age influences the accuracy of RSBs for diagnosing HD disease. The sensitivity of the RSB outcomes, in particular, was significantly lower when the RSBs were obtained in patients younger than 39 days. Moreover, the probability of a correct HD diagnosis increased gradually with increasing age and reached a maximum at approximately 125 days (Figure 3A). In contrast, the specificity of RSB outcomes was not influenced by age and remained high (95%) in all the age groups we investigated.

Several factors can influence the sensitivity of RSB outcomes, like varying biopsy sites, technical issues to do with the staining procedures, and the amount of experience of the pathologist. These factors, however, do not account for the difference in sensitivity in relation to age. We believe that the influence of age resulted from the immaturity of the enteric nervous system, which is still dynamically developing, and therefore quickly changing after birth. This aspect was also mentioned by Nakao and colleagues,[10] who demonstrated that nerve fibers continue to proliferate even after a child is born with HD. Thus, the developmental characteristics of AChE activity, related to changes in

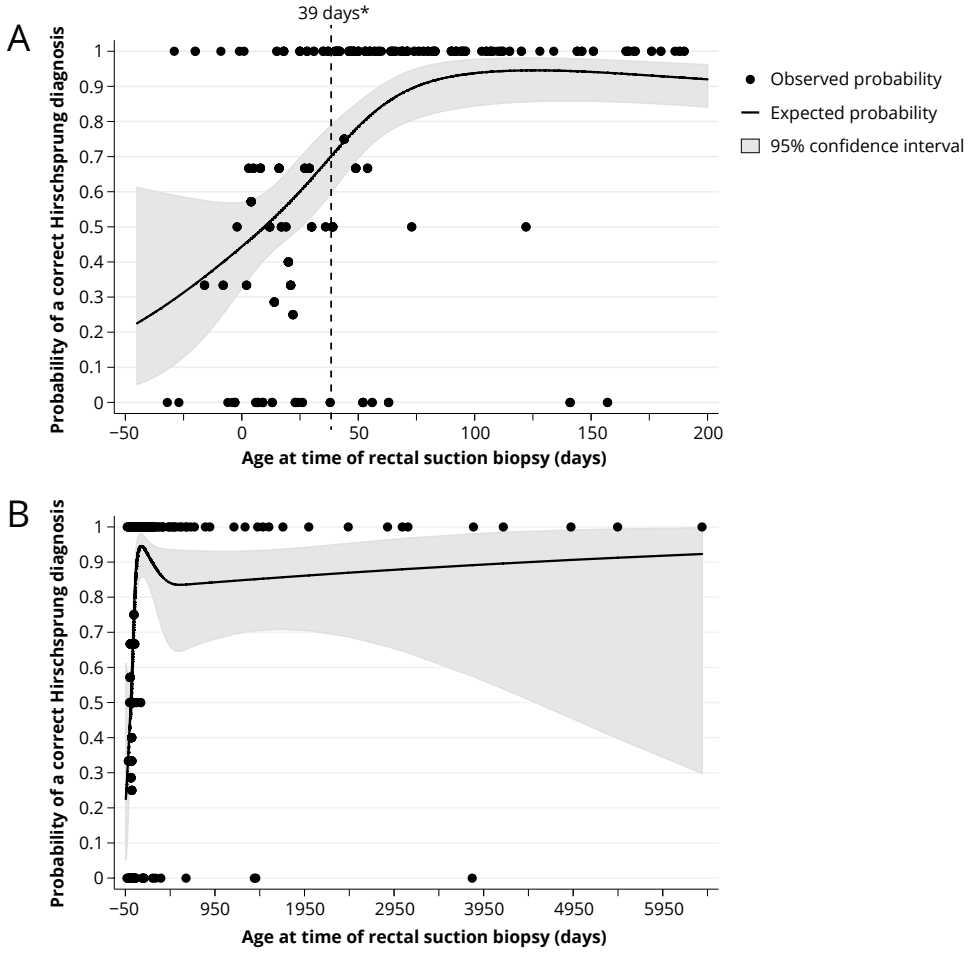

Figure 3

The probability of a correct HD diagnosis plotted against the age at the time of the RSB. Probability was defined as the number of true positive RSB outcomes.

A: RSBs obtained at the ages -45 to 200 days (corrected for gestational age). Probability increased and reached a maximum of 0.95 at the age of approximately 125 days.

B: RSBs obtained at the ages -45 to 6390 days (corrected for gestational age). A slight decrease of probability down to 0.85 was observed after the initial increase at the youngest age.

* The line at 39 days depicts the cutoff point at which we observed a significant difference in the sensitivity between groups younger than 39 days and groups older than 39 days ($P < .001$).

the density of nerve fibers, results in a different staining pattern in younger patients before the classic pattern, characteristic of HD as seen in older patients, has had time to develop.[16] On the one hand one could, therefore, argue that AChE enzyme histochemistry should be reconsidered since it may be responsible for the lower sensitivity of RSB outcomes in younger patients. On the other hand, however, it was reported that AChE enzyme histochemistry greatly increases the specificity of RSB outcomes and reaches a maximum of 98%.[17] Our results do indeed confirm that staining with AChE enzyme histochemistry, combined with NADH enzyme histochemistry and routine H&E staining, resulted in a high average specificity of 95% in all the age groups we investigated. The specificity we found was comparable to the findings reported by other authors.[3,18,19] In addition, we demonstrate that age does not have a statistically significant influence on the specificity of RSB outcomes. Nevertheless, we did observe that there remains a small risk of obtaining a false positive RSB outcome that could lead to an incorrect diagnosis, redundant treatment, and potentially even to unnecessary surgical intervention.

Interestingly, it has been reported that insufficient tissue required for analysis was obtained significantly more often in the case of RSBs in patients who were older than three years.[20] The insufficiency of material in the older group of patients might be a result of the fact that submucosal tissue in older children is more fibrous, which makes it more rigid and, therefore, more difficult to extract through suction.[21] In our study, we indeed observed a steady decrease of sensitivity after the age of 125 days (Figure 3B), a finding which could be explained in terms of the aforementioned reason. Nevertheless, the RSBs obtained from patients older than 125 days, were not deemed inadequate by the pathologist because insufficient tissue had been extracted. Instead, these were classified as inconclusive for diagnosing HD accurately. It would, therefore, seem important to critically define the quality of RSBs in older patients, especially if sufficient submucosal tissue was obtained to assess the presence of ganglion cells.

Furthermore, we found that the RSBs that were excluded due to insufficient tissue, were obtained from patients of a statistically significant younger age than the RSBs that yielded sufficient tissue (43 days in insufficient biopsies versus 78 days in sufficient biopsies, $P = .033$). A reason for this discrepancy remains unclear. Also, RSBs yielding insufficient tissue were seen significantly more often in non-Hirschsprung patients than in HD patients (8.3% versus 3.0%, $P = .013$). This difference might be explained by the fact that technically it is easier to perform RSBs in HD patients due to constant contractions of their rectal wall. Additional studies are required, however, to confirm this theoretical explanation.

As mentioned in the results, a total of 12 RSB taken from 11 patients were inconclusive for HD, with the advice of the pathologist to repeat the RSB. Possibly, the biopsies were

obtained from a faulty location. It is generally accepted that the most distal 1 to 2 cm of the rectum are hypoganglionotic.[22] Accidental RSBs at this height could lead the pathologist to misdiagnose an ultra-short variant of HD.

In our study only one patient had a false positive diagnosis of HD. Anorectal manometry at the age of 18 years revealed the presence of a functioning rectoanal inhibitory reflex. It is generally accepted that the absence of this reflex is a distinguishing feature of HD.[1] It has been postulated that false positive and inconclusive RSB outcomes in healthy children might be due to unspecific AChE staining of AChE in red blood cells membranes in hemorrhagic tissue.[23,24] As a consequence, AChE staining performed on tissue obtained from a hemorrhagic specimen could be unspecific and, therefore, lead to the misinterpretation of RSB outcomes.

Possible clinical implications

Based on our study and literature, we reckon that RSBs obtained from patients younger than 39 days (corrected for gestational age) should be analyzed with the utmost care since it seems that there is no gold standard below this age. Since in the youngest patients RSB outcomes reached a maximum sensitivity of 62%. Moreover, obtaining RSBs is an invasive technique with a small risk of complications. In case of suspected HD, therefore, we would rather choose a noninvasive technique to make the primary diagnosis. Subsequently, a RSB could be obtained to confirm this diagnosis once the infant is older than 39 days. None of the conventional tools currently used to diagnose HD are ideal. Nevertheless, since anorectal manometry is a noninvasive technique and has little to no adverse effects, we propose using it as the screening tool in patients younger than 39 days of age. By so doing, an invasive RSB is avoided if a functioning rectoanal inhibitory reflex is found by anorectal manometry.

Conclusions

As we hypothesized, the outcomes of RSBs obtained below the age of 39 days have a significantly lower sensitivity for diagnosing HD. Additionally, RSB obtained from younger patients often lead to inconclusive outcomes and require additional biopsies. If possible, performing RSBs in patients younger than 39 days should be avoided and, if the RSBs obtained below this age are negative or inconclusive for HD, they should be repeated if the symptoms of the patient persist.

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