Section: Pathology

Original Research Article

COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY CALRETININ IN HIRSCHSPRUNG DISEASE IN A PEDIATRIC TERTIARY CARE CENTER

M.S. Muthu Prabha[1], S. Preetha[2], R. Vedamoorthy[3], A. Peter Samidoss[4], V. Buvaneswari[5]

[1]Assistant Professor, Department of Pathology, Government Theni Medical College, Theni, India
[2]Assistant Professor, Department of Pathology, Madras Medical College, Chennai, India
[3]Assistant Professor, Department of Pathology, Government Theni Medical College, Theni, India
[4]Assistant Professor, Department of Pathology, Government Theni Medical College, Theni, India
[5]Assistant Professor, Department of Pathology, Government Theni Medical College, Theni, India

Received : 05/12/2024
Received in revised form : 28/01/2025
Accepted : 13/02/2025

Keywords:
Hirschsprung disease, Ganglion cells, Toluidine blue.

Corresponding Author:
Dr. V. Buvaneswari,
Email: drbuvanavinoth@gmail.com

DOI: 10.47009/jamp.2025.7.1.231

Source of Support: Nil,
Conflict of Interest: None declared

Int J Acad Med Pharm
2025; 7 (1); 1189-1196

Abstract

Background: Hirschsprung disease, a congenital disorder with an incidence of 1/5000 live births and is common in male infants (85%). Histopathologically absence of ganglion cells is diagnostic of Hirschsprung disease, but presence of submucosal hypertrophic nerve fibers is an additional positive finding.This study is to evaluate the diagnostic efficacy of toluidine blue special stain in diagnosing Hirschsprung disease when compared to immunohistochemistry–calretinin. **Materials and Methods:** It is a two years' study in a histopathologically diagnosed cases of Hirschsprung disease. In 95 biopsy proven cases of disease, 55 were analysed with Immunohistochemistry-Calretinin and special stain Toluidine blue. Also Immunohistochemistry S100 also done for supporting the diagnosis of Hirschsprung disease. **Result:** On immunohistochemical evaluation with Calretinin, 3 out of 55 cases showed positivity which implies that there is presence of ganglion cells. Special stain Toluidine blue showed positivity in 3 cases where ganglion cells were present in which Calretinin was also positive. The results with Calretinin and Toluidine blue are comparable and the results are statistically significant (< 0.001). **Conclusion:** To conclude, histopathology alone will not help in confirming the diagnosis of Hirschsprung disease as immature ganglion cells are difficult to identify in histopathology. Immunohistochemistry can be used when there is difficulty in identifying ganglion cells. Special stain Toluidine blue can be used as an alternative to immunohistochemistry in places where Immunohistochemistry not available.

INTRODUCTION

The disease was named after Harald, a Danish paediatrician, the first person to describe the condition of two unrelated boys who died from chronic severe constipation with abdominal distension resulting in congenital megacolon in the year 1888 (Amielet al., 2008).[1] In the year 1940, Tiffin and associates described the local absence of ganglion cells in the myenteric plexus in a patient with congenital megacolon with ganglion cells present above and below the lesion (Whitehouse and Kernohan, 1948).[2] The condition leads to loss of tonic neural inhibition, persistent contraction of the affected segment, and subsequent colonic obstruction. The condition is usually due to defective craniocaudal migration of vagal neural crest cells (the progenitors of ganglion cells) between gestational weeks 5 and 12 (Tiffinet al., 1940).[3] Interruption of cranio-caudal migration of neural crest cells explains distal aganglionosis in Hirschsprung disease. The aganglionic segment in Hirschprung disease begins at the anal sphincter and extends proximally. The diagnosis of Hirschsprung disease is supported by barium enema studies, anorectal manometry, and rectal biopsies. The gold standard method is the microscopic evaluation of rectal biopsies. The absence of ganglion cells in an adequate biopsy is diagnostic of disease (Marielleet al., 2015).[4] In addition, there might be an increase in non-myelinated cholinergic nerve fibres in the sub-mucosa and among muscle layers (neural hyperplasia), which helps in diagnostic confirmation. Calretinin stains ganglion cells and Calretinin positivity rules out disease (Vincent et al., 2009; Holland et al., 2011).[5,6] S100 immunostain can highlight the presence of hypertrophic submucosal

ISSN (O): 2687-5365; ISSN (P): 2753-6556

nerve fibres (Luis and Karla, 2012).[7] Toluidine blue stain is a synthetic, acidophilic metachromatic dye that has an affinity for nucleic acids and therefore binds to nuclear material with a high DNA and RNA content, in chromatin or Nissl substance and selectively stains nucleus blue and cytoplasm light blue. This stain is used to identify the ganglion cells (Hadeel, 2015).[8]

Surgery is the treatment of choice for Hirschsprung disease. It also prevents further complications of enterocolitis (Jennifer, 2006).[9] This study is to assess the efficacy of Toluidine blue staining in the detection of ganglion cells and confirm with Immunohistochemistry (IHC). In centres where Immunohistochemistry is not readily available, Toluidine blue can be used as a cost-effective substitute for Immunohistochemistry. Careful clinical examination is required. To establish the diagnosis, several diagnostic studies should be performed. Plain abdominal radiographs may show distended bowel loops with absent air in the rectum (Pensabeneet al., 2003).[10]

Aim

To evaluate the diagnostic efficacy of Toluidine blue special stain in diagnosing Hirschsprung disease when compared to Immunohistochemistry in Calretinin and also Immunohistochemistry S 100 helping to support the diagnosis of Hirschsprung disease.

MATERIALS AND METHODS

In this study, we performed both prospective and retrospective data analysis of patients who were diagnosed to have biopsy-proven Hirschsprung disease over a period of two years in a paediatric tertiary care centre.

During our study period, we received around 2,524 specimens for histopathological examination. Since it is a paediatric hospital we received 400 specimens from the gastrointestinal tract out of which we received 110 cases of clinically suspicious Hirschsprung disease. Out of 110 cases, 95 cases were Hirschsprung disease, one case was intestinal neuronal dysplasia,8 cases as non-Hirschsprung disease, and for 6 cases opinion could not be given. Out of 95 cases, 55 cases were taken for to do both Immunohistochemistry and special stain.

Inclusion Criteria

- Cases of Hirschprung disease diagnosed by Histopathological examination by Haematoxylin and Eosin
- Cases of age group less than 5 years.

Exclusion Criteria

- Blocks with inadequate material.
- Other causes of aganglionosis.
- Age group more than five years.

Method of Data Collection

Detailed history of the cases regarding age, sex, complaints like delayed passage of meconium, abdominal distension, constipation, and failure to thrive, imaging findings were obtained for 110 cases reported during the study period from the surgical pathology records. All the specimens were processed and representative sections were taken and subjected to routine histopathological examination. The following clinical and pathological parameters were evaluated age, gender, complaints, and site of the lesion.

Hirschsprung disease was typed according to presentation confirmed by imaging and pre-operatively. Absences of ganglion cells are viewed meticulously since biopsies are received from 3 sites from the same patient. The sites are the transition zone, proximal colostomy site, and distal colostomy site. The absence of ganglion cells confirms the diagnosis of Hirschsprung disease. While reporting the presence of ganglion cells in the colostomy site should be given, because the presence of ganglion cells is required for the proper functioning of colostomy stoma.

Immunohistochemistry was done with Calretinin and S100. A special stain was done by using Toluidine blue for the same cases.

Immunohistochemical Evaluation:

Immunohistochemical analysis of Calretinin & S100 was performed in paraffin-embedded tissue samples by using a super sensitive polymer HRP(horse radish peroxidase) system based on non-biotin polymeric technology.

4-micron sections were cut from formalin-fixed paraffin-embedded tissue samples and transferred onto positively charged slides. Heat-induced antigen retrieval method was used. The antigen was bound with rabbit polyclonal antibody against Calretinin and rabbit monoclonal antibody against S100 protein and then detected by adding a secondary antibody conjugated with horse radish peroxidase-polymer and diaminobenzidine substrate.

Interpretation of IHC

The antibody-treated slides were analyzed for the presence or absence of reaction, localization of the staining pattern, percentage of cells stained, and intensity of the reaction.

Calretinin shows strong cytoplasmic positivity in ganglion cells. The presence of ganglion cells excludes the diagnosis of Hirschsprung disease.

S100 shows strong cytoplasmic positivity in the sub-mucosal nerve trunk. Focally it will be positive in normal nerve fibres, but if there are thickened nerve trunks they are a hypertrophic nerve bundle which is an additional criterion for diagnosing Hirschsprung disease. Thickness could be of>40µ.

Special stain

Toluidine blue staining in paraffin sections was performed using a simple method that required incubation of sections in 0.2% aqueous solution of Toluidine blue at 56oC for 30 minutes and finally mounting in a water-based medium.

Interpretation of cytoplasm stains an ultramarine blue color and nucleus blue color. This is due to the presence of Rough endoplasmic reticulum which is present in the Nissl substance which is present in the cytoplasm.

ISSN (O): 2687-5365; ISSN (P): 2753-6556

Statistical Analysis: The statistical evaluation was performed with the IBM-SPSS statistical package for the social sciences version 20. An initial analysis of collected variables was performed. Immunohistochemical expression of Calretinin, S100 antibody, and special stain Toluidine blue was analyzed and correlated with clinical variables like age, sex, clinical presentation and pathological variables like absence of ganglion cells and hypertrophic nerve bundles in histopathological sections.

Pearson Chi-square test was used in analyzing these variables. Immunohistochemical expression of Calretinin and S100 are reported. Calretinin expression was compared with Toluidine blue and analyzed for statistical correlation and a P value below 0.05 is considered significant.

RESULTS

During our study period of 24 months, we received around 2,524 specimens for histopathological examination in a paediatric tertiary care centre. Of this, 110 cases of clinically suspicious Hirschsprung disease both colostomy and biopsy specimens were received. In these,8 suspected cases was found to be a Non-Hirschsprung disease showing the presence of ganglion cells [Figure 1A]. One case which was suspected as Hirschsprung disease was found to be a case of intestinal neuronal dysplasia. 3 cases had inadequate material for processing and 3 cases showed only the mucosa from which a conclusive opinion cannot be given because only Hirschsprung disease is diagnosed mainly in the sub mucosa, 95 cases showed the absence of ganglion cells diagnosed to be Hirschsprung disease. From this 95 cases of biopsy proved Hirschprung disease, and 55 were taken for the study and analyzed [Table 1].

Age-wise distribution of among 55 cases taken up for analysis,34 cases are from the neonatal period, 10 cases were from 29 days to one year of age,6 were under 1 to 3 years of age and 5 cases were under 3 -5 years of age group. In total 44 cases were less than one year of age group. Cases from later age groups have reduced incidence. Age incidence of 55 cases of the study 62% were within the first 28 days of life which is the neonatal period [Table 2].

Sex-wise distribution of among 55 cases of Hirschsprung disease,49 cases were male children and the remaining 6 were female cases. This finding correlates with literature where male patients predominate in disease manifestation. Among the 55 cases, 89% cases were male children and the remaining 11% were female children [Table 2].

Clinical presentation of 55 cases and the majority of cases were newly born presenting with complaints of delayed passage of meconium. The next mode of presentation in the newborn was abdominal distension. Constipation is the presenting complaint in children of age more than one year. Delayed passage of meconium and abdominal distension is found to be the most common presentation of Hirschsprung disease since it is a disease that is presented in the early neonatal period. Constipation is the complaint of older children which would be the late manifestation of the disease. Other complaints of Hirschsprung disease are failure to thrive and enterocolitis [Table 2].

Of the 55 cases of Hirschsprung disease, approximately 48 presented as a short-segment disease with rectosigmoid involvement. The next most common presentation was long-segment disease. Finally, we had two cases of total aganglionosis which is associated with a very grave prognosis. The most common type encountered in our study was short-segment disease with a rectosigmoid presentation followed by long-segment disease. Two cases of total aganglionosis were seen with involvement of the total bowel and were associated with a grave prognosis [Table 2].

Among the 55 cases of Hirschsprung disease, 43 patients underwent a colostomy procedure, 5 patients received the Duhamel pull-through procedure, and 7 cases involved a seromuscular biopsy. Seromuscular biopsy was done when there is a strong clinical suspicion of disease. Duhamel pull-through is done after the colostomy procedure in a period of gap. Among 55 cases 78% of cases were from colostomy procedure and 9% from Duhamel pull through and around 13% were seromuscular biopsy [Table 2].

For imaging findings, all the cases present with delayed passage of meconium, and further evaluation was done by imaging. Initial imaging was done by both ultrasonogram and x-ray, further confirmed by barium contrast study. In ultra-sonogram and x-ray, the common finding is the dilated bowel loops.In our hospital nearly all cases were diagnosed by x-ray findings with dilated bowel loops [Table 3]. Initial diagnosis was made by histopathological examination using H&Estained paraffin sections by absence of ganglion cells [Table 3]. Immunohistochemistry was initially not done.

A hypertrophic nerve bundle in histopathological examination of the presence of hypertrophic nerve bundles was an additional finding in Hirschsprung disease [Figure1B]. However, its absence does not exclude the diagnosis. When it is present along with the absence of ganglion cells it helps in the diagnosis of disease. In our study, we had hypertrophic nerve bundles seen in 47 cases and only 8 cases showed the absence of hypertrophic nerve bundles [Figure 5].

Immunohistochemical expression of Calretinin out of 55 cases of HPE diagnosed Hirschsprung disease, 3 cases were positive for Calretinin. Calretinin is a marker of ganglion cells. So it showed that ganglion cells were present in these cases which were overlooked in HPE. Immunohistochemistry confirms the presence of ganglion cells since was positive. Among the 55 cases of histopathologically diagnosed Hirschsprung disease, 3 cases showed Calretinin positive which is about 5.5% in total. Calretinin is a marker of ganglion cells (Figure 2A to 2D). It confirms the presence of ganglion cells which is

ISSN (O): 2687-5365; ISSN (P): 2753-6556

against the histopathological diagnosis [Table. 4]. Remaining cases were Calretinin negative [Figure 2E].

Immunohistochemical Expression of S100 indicates the presence of nerve fibres, positivity denotes there are thickened nerve bundles [Figure 3 A,B] and focal positive (Fig. 3D) denotes there are no thickened nerve bundles just nerve fibres are present. S100 also highlights the ganglion cells by negative staining surrounded by Schwann cells and glial fibres [Figure 3C]. Out of 55 cases, 47 cases showed positivity which is the thickened nerve bundles. 6 cases showed focal positivity denoting the presence of nerve fibres and in 2 cases S100 was negative. Out of 55 Cases, 85% showed positive, 11% showed focal positivity and 4% were negative. It is not a confirmatory test and it is just an additional finding [Table 4].

Expression of a special stain Toluidine blue was also used here for the identification of ganglion cells. In 55 cases, 3 cases showed positivity [Figure 4A,B], the same cases where Calretinin was positive which also helps in diagnosing ganglion cells. In sections stained with Toluidine blue mast cells are also seen. Toluidine Blue special stain showed 5.5% positivity [Table: 4].

Comparison of Calretinin with Toluidine blue the Calretinin positive in 3 cases and Toluidine blue positive in the same 3 cases. Both stains aid in identifying the ganglion cells [Figure 6]. And comparison of Calretinin and S100, Calretinin negative in 52 cases and S100 positive out of 53 cases [Figure 7].

The results with Calretinin and Toluidine blue are comparable and the results are statistically significant (< 0.001) and 100% sensitive but specificity cannot be arrived since it needs larger samples. If the number of cases increases with larger samples then it can be concluded that Toluidine blue is a better method. But in our conclusion, Toluidine blue is equally good as calretinin. It can be used as a substitute for Calretinin in cases where it is unavailable [Table 5].

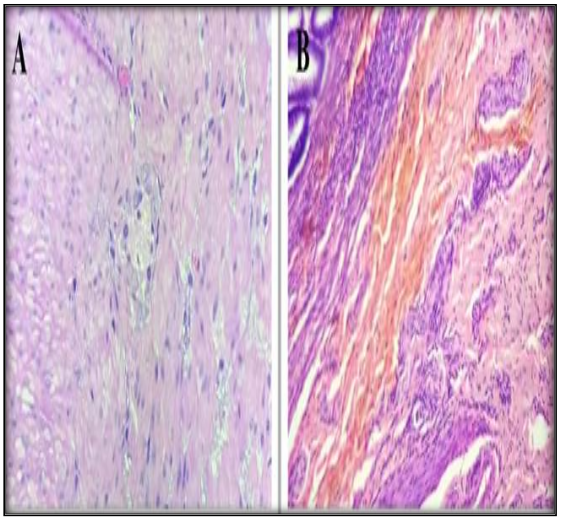

Figure 1: (A).H&E staining showing presence of Ganglion Cells (400X), (B). H&E staining showing Nerve bundle hypertrophy (100X)

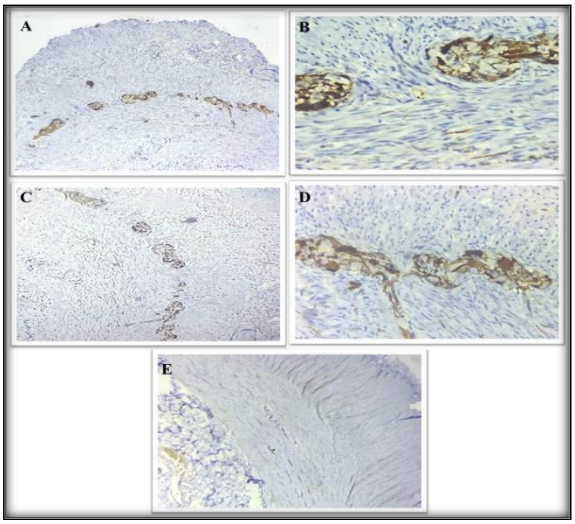

Figure 2: (A). IHC showing Calretinin Positive (100X), (B). (400X),(C). (100X), (D). (400X), (E). IHC showing Calretinin Negative (100X)

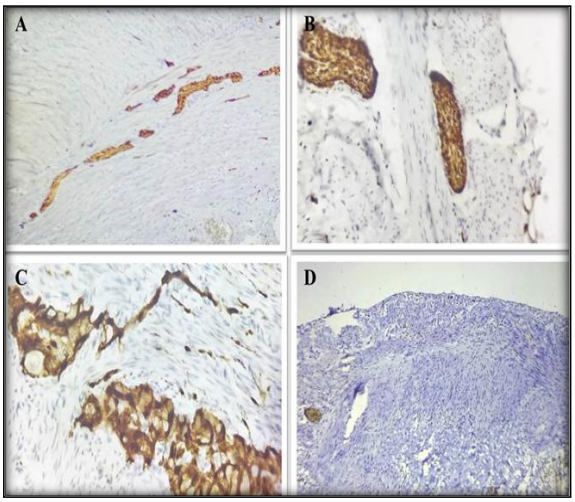

Figure 3: (A). IHC showing Focal S100 Positive (100X), (B). IHC showing Thickened Nerve Bundles in S100 (400X). (C). IHC S100 Negative Staining of Ganglion Cells (400X). (D). IHC showing Focal S100 Positive (100X)

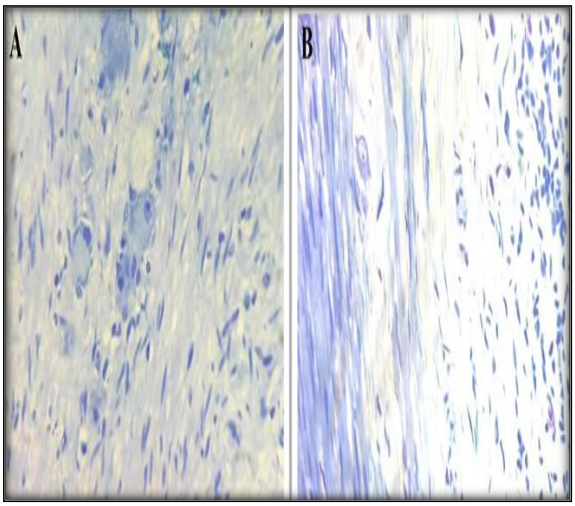

Figure 4: (A). Toluidine Blue stain showing positive in Ganglion Cells (400X), (B). Toluidine BlueStain Showing Positive in Ganglion Cells (400X)

ISSN (O): 2687-5365; ISSN (P): 2753-6556

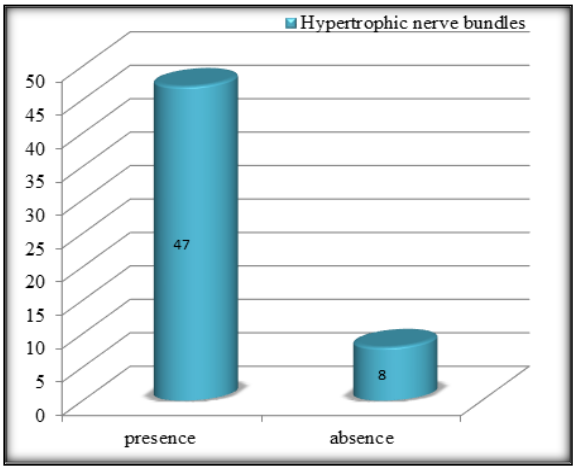

Figure 5: Showing Hypertrophic Nerve Bundles

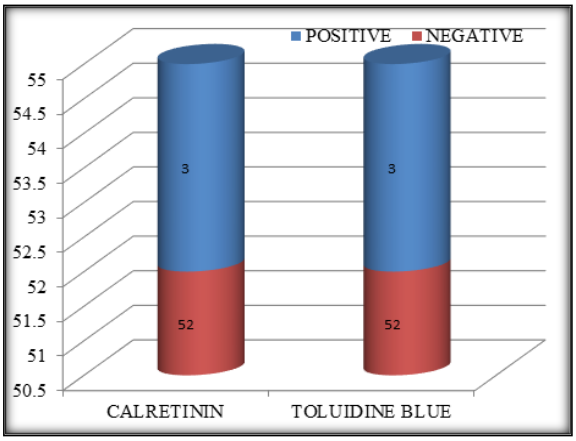

Figure 6: Showing Comparison of Toluidine Blue with Calretinin

Table 1: Specimens received from clinically suspicious Hirschsprung disease (110)..

Clinical Suspicious	Diagnosis
Hirschsprung Disease	95
Non Hirschsprung	8
Intestinal Neuronal Dysplasia	1
Inadequate Material	3
Inconclusive Opinion	3
Total	110

Table 2: Showing Age wise distribution of cases (55 cases study), Sex wise distribution, The Clinical Complaint Presentation, Types of Hirschsprung Disease and The types of procedure done for Hirschsprung disease.

	Frequency	Percentage (%)
Age wise distribution of cases (55 cases study)		
0-28 Days	34	62
29 Days -1 Year	10	18
1 Years - 3 Years	6	11
3-5 Years	5	9
Total	55	100
Sex wise distribution		
Male	49	89
Female	6	11
Total	55	100%
The Clinical Complaint Presentation		
Delayed passage of Meconium	30	55
Abdominal Distension	5	9
Constipation	20	36
Types of Hirschsprung Disease		
Short Segment HD	48	87%
Long Segment	5	9%
Total Aganglionosis	2	4%
The types of procedure done for Hirschsprung disease		
Biopsy	7	13%
Colostomy	43	78%
Duhamel- pull through	5	9%
Total	55	100%

Table 3: Showing Tabular Column of Imaging Findings and Absence of ganglion cells.

	Frequency	Percent	Valid (%)	Cumulative (%)
Tabular Column of Imaging Findings (Dilated bowel loops)	55	100.0	100.0	100.0
Absence of Ganglion Cells	55	100.0	100.0	100.0

Table 4: Showing Immunohistochemical Expression of Calretinin, S100 and Toluidine blue.

	Frequency	Percentage (%)
Immunohistochemical Expression of Calretinin		
Negative	52	94.5
Positive	3	5.5
Total	55	100.0
Immunohistochemical Expression of S100		
Focal positive	6	11
Negative	2	4
Positive	47	85

ISSN (O): 2687-5365; ISSN (P): 2753-6556

Total	55	100
Toluidine Blue Expression		
Negative	52	94.5
Positive	3	5.5
Total	55	100

Table 5: Comparison of Calretinin and ToluidineBlue

			Calretinin		Total
			Positive	Negative	
Toluidine Blue	Positive	Count	3	0	3
		% Within Calretinin	100.0%	.0%	5.5%
	Negative	Count	0	52	52
		% Within Calretinin	.0%	100.0%	94.5%
	Total	Count	3	52	55
		% Within Calretinin	100.0%	100.0%	100.0%

[Table 5] infers that Toluidine Blue is positive in 100.0% of the CALRETININ-positive group, with no negative results. The CALRETININ-negative group is 100.0% negative for Toluidine Blue, with no positive instances. Overall, 5.5% of the samples tested positive for Toluidine Blue, whereas 94.5% were negative.

Since all Toluidine Blue-positive cases are only identified in the CALRETININ-positive group, there is a perfect correlation between CALRETININ positivity and Toluidine Blue positivity. In the same way, every case that is CALRETININ-negative is also Toluidine Blue-negative. Given this substantial link, it is possible that Toluidine Blue positive could be a trustworthy indicator of CALRETININ positivity in this dataset.

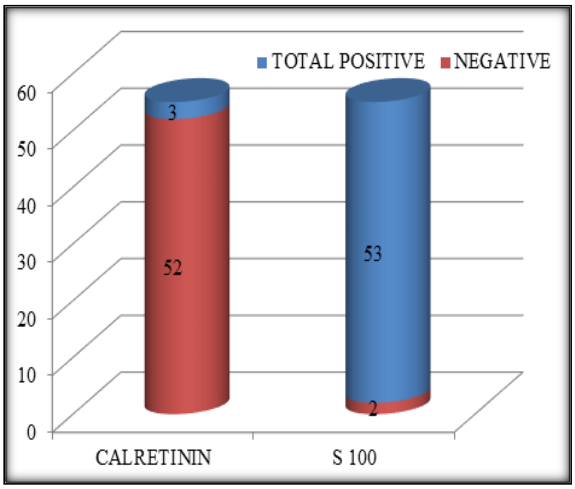

Figure 7: Showing Comparison of Calretinin with S100

DISCUSSION

Hirschsprung disease is common in new-borns and it is more common in male children presenting more frequently in less than 1 year of age group. Similar to many other studies our study also had 49 out of 55 cases presenting in less than one-year age group. Spouge and Baird (1985) showed that new born males were most commonly affected by disease. Goldberg (1984) showed similar results. Ikeda and Goto (1984) studied 1628 cases of disease and concluded that males are more commonly affected than females. The most common presentation is delayed passage of meconium. Some cases present with abdominal distension. In our study around 55% of patients presented with delayed passage of meconium.

Holschneideret al., (1994) described short-segment disease as the most common presentation.[11] In our study also 87.3% were of short segment type. In our study as our institution is a paediatric hospital we had a variety of presentations of Hirschsprung disease. Out of 110 clinically suspected cases of Hirschsprung disease, only 95 were histopathologically proven Hirschsprung disease. We included cases taken cases of disease in age groups less than 5 years and we analysed about 55 cases. 55 cases have been analyzed both by using Immunohistochemistry and special stain. In Immunohistochemistry, Calretinin and S 100 was used. Calretinin to identify ganglion cells and S100 to stain the nerve fibres, since hypertrophic nerve bundle is an additional finding but it is not diagnostic.

Immunohistochemical expression of Calretinin in Hirschsprung disease highlights the presence of ganglion cells. Calretinin positivity confirms the diagnosis of non-Hirschsprung disease. Calretinin is both a sensitive and specific marker for diagnosing Hirschsprung disease. In a study by Barshacket al., (2004),[12] 54 paraffin blocks were taken 24 from the ganglionic segment, 17 from the aganglionic segment, and 13 from the transitional zone.10 out of thirteen cases showed ganglion cells with Calretinin positivity, which is around 80%. Zuikovaet al,[13] (2015) performed a study on40 cases of Hischsprung disease and it showed a sensitivity of 100% to Calretinin.

Małdyket al,[14] (2014) reported a study in 2014 in about 14 cases of suspected Hirschsprung disease 11 cases showed the absence of ganglion cell positivity and was confirmed by Calretinin immunostaining and it showed 99.1% sensitivity. Vincent et al,[15] (2009) studied in nearly 130 cases and showed nearly 100% sensitivity. Hiradfaret al,[16] (2012) studied 50 cases and showed a sensitivity of 93.3%. Mukhopadhyay et al,[17] (2015) reported that the sensitivity of Calretinin Immunohistochemistry for ganglion cells detection was 100% and they studied 105 cases. Kaçaret al,[18] (2012) showed Calretinin

ISSN (O): 2687-5365; ISSN (P): 2753-6556

was found to be highly sensitive and specific in diagnosing Hirschsprung disease in their study in 43 cases.

Gonzalo and Plesec (2013) found in their retrospective study that all patients showed negative Calretinin expression concluding that immunohistochemical testing of Calretinin is quite supportive in triaging additional workup based on clinical suspicion in a 48 case study with 40 cases of Hirschsprung disease. Calretinin was extremely useful in solving the suspicious and indeterminate cases of HD. It can serve as a valuable cost-effective diagnostic aid in centers where acetylcholinesterase enzyme histochemistry is not available (Lavanya et al., (2013).[19]

In our study, we had 55 histopathologically proved cases of Hirschsprung disease,but in it we had 3 cases of Calretinin positivity that is which showed the presence of ganglion cells. Based on these studies keeping Calretinin IHC as the gold standard test it has been compared with Toluidine blue special stain. We demonstrated three cases of Calretinin positivity out of 55 cases of HPE proved Hirschsprung disease.

A false positive interpretation of HD in biopsy can be given due to the following factors such as 1. Minimally appreciable submucosa depending on the biopsy obtained, 2. Inappreciable and infrequent ganglion cells in submucosa, 3. Difficulty in identifying immature ganglion cells in neonates and 4. Observer error due to lack of experience. If Calretinin is positive in the biopsy of the suspected case, then it excludes Hirschprung disease. When it is present in the colostomy site pathologists are given a piece of additional information that colostomy would function well helping in good prognosis of the patient. So it is more important to know the site of the specimen received.

Immunohistochemical expression of S100 is not used as a diagnostic tool for Hirschsprung disease but can be used as an additional criterion for diagnosing disease. Since S100 stains the nerve fibres,it was used to identify the thickened nerve bundles.S100 also highlights the ganglion cells by negative staining surrounded by Schwann cells and glial fibres. In Holland et al,[20] (2011) studies 138 cases were studied and 81% showed thickened nerve fibres. In Luis et al,[21] (2012) studied 14 cases with each having 50 section showed low sensitivity of 41.7%. In a study by Barshacket al,[22] (2004) 54 paraffin blocks were taken 24 from the ganglionic segment, 17 from aganglionic segment and 13 from the transtitional zone,10 out of thirteen cases showed ganglion cells presence with Calretinin positivity, which is around 80% also S100 showing thickened nerve bundles with 100% sensitivity.

Toluidine blue special stain is the one used for identifying mast cells by the property of metachromasia, but in one study in a paediatric hospital, Toluidine blue has been used as a stain for identifying ganglion cells. Since Toluidine blue is a cheap and easy method this has been chosen for the studies to identify the ganglion cells. Hadeelet al,[23] (2015) studied in a total of 50 non-selected cases biopsied for suspected HD which was stained with H&E stain. Based on the findings of H&E stained sections of rectal biopsies, they were divided into two groups such as HD included 20 cases (40%) and non-HD included 30 cases (60%). By using Toluidine blue special stain the ganglion cells were identified in 34 (68%) out of 50 cases and the ganglion cells were very easily identified in 36% of cases. Canilet al., (2007) their study stated that the Toluidine blue method is a reproducible and reliable way of demonstrating ganglion cells in frozen rectal biopsies. Based on these two studies, Toluidine blue special stain was taken into our study and has been compared with that of Immunohistochemistry. By having Immunohistochemistry as a gold standard, results are compared with the results of Toluidine blue. Toluidine blue is positive in three cases where the IHC Calretinin was also positive. In comparison, both showed similar results. As per statistical analysis also it is statistically significant showing p value <0.001. Despite similar results, Toluidine blue cannot be said as superior to IHC. Sensitivity is good but to know the specificity we need studies in a large sample. The study concludes that Toluidine blue can be used as an alternative for IHC in centres where Immunohistochemistry is not available.

Summary

For the study period of two years, a total of 2,564 specimens were received in the pediatric tertiary care centre for histopathological examination. 95 cases of Hirschsprung disease have been confirmed by histopathological examination out of 110 clinically suspicious cases.34 out of 55 cases were present in the first 28 days of life (i.e.neonatal period). 44 cases out of 55 were present in the first year of life.

Male children accounted for 90% of the cases with female children accounting for 10% of cases. 64% of cases presented with delayed passage of meconium and abdominal distension.87% of cases presented as a short-segment disease.

2 out of 55 cases were total aganglionosis type of Hirschsprung disease. 78% of cases were had colostomy for Hirschsprung disease. All clinically suspicious cases of Hirschsprung disease underwent imaging which showed dilated bowel loops. In 55 cases selected the histopathology was reported as the absence of ganglion cells. Out of 55 cases, 47 cases showed hypertrophic nerve bundles in the submucosa.

On immunohistochemical evaluation with Calretinin 3 out of 55 showed positivity which implies that there is presence of ganglion cells. In the immunohistochemical evaluation of S100, out of 55 cases, 47 cases showed thickened positivity, 6 cases showed focal positivity and 2 cases were S100 negative.

Special stain Toluidine blue showed positivity in 3 cases where ganglion cells were present. The same cases showed Calretinin positivity in ganglion cells. The results with Calretinin and Toluidine blue are comparable and the results are statistically significant

ISSN (O): 2687-5365; ISSN (P): 2753-6556

($p<0.001$). 100% sensitive but specificity cannot be arrived at since it needs larger samples. If the number of cases increases with larger samples then we can conclude that Toluidine blue is a better method.
On follow-up of the cases of the Hirschprung has given the conclusion that long segment and total aganglionosis had a very grave prognosis of having high mortality.

CONCLUSION

To conclude, Hirschsprung disease is a disorder presenting in males most commonly presenting as a short segment. Detection of ganglion cells is a diagnostic challenge in histopathology since immature ganglion cells of neonates are very difficult to identify.
Hence histopathology alone will not help in confirming the disease. The serial section of a biopsy should always be done for interpretation. A minimum of 20 sections should be given to confirm the diagnosis of Hirschsprung disease.
Immunohistochemistry can be used when there is difficulty and strong suspicion of the presence of ganglion cells. Special stain Toluidine blue also can be used in places where Immunohistochemistry is not available. Though Toluidine blue special stain had valid significant results it should be studied extensively with a larger sample size. Since Toluidine blue special stain is cheap, easily available, and not time-consuming it can be used as an alternative for Immunohistochemistry.

Acknowledgments
The authors are supported by the Department of Health Research (DHR) from the Multi-Disciplinary Research Unit (MDRU) of Government Theni Medical College & Hospital, Theni, Tamilnadu during manuscript preparation, data analysis, and scientific communications phase.

REFERENCES

1. Amiel J,E Sproat-Emison, M Garcia-Barcelo, F Lantieri, G Burzynski, SBorrego, APelet,SArnold,XMiao,PGriseri,ASBrooks,GAntinolo, L de Pontual, M Clement-Ziza, A Munnich, C Kashuk, K West,K K-Y Wong, S Lyonnet, A Chakravarti, P K-H Tam, I Ceccherini, R MWHofstra,RFernandez. (2008). Hirschsprung disease, associated syndromes and genetics: areview. JMedGenet.45(1):1-14..
2. BarshackI,FridmanE,GoldbergI,(2004).Theloss of Calretinin expression indicates aganglionosisHirschsprung's disease. J ClinPathol57(7): 712-716.
3. Canil M,K.Meir,G.Jevon,T.Sturby,S.Moerike,A.Gomez. (2007). ToluidineBlueStainingisSuperiortoH&EStainingforthe Identification of Ganglion Cells in Frozen Rectal Biopsies. TechnicalbulletinforHistotechnology.1:1-3.
4. Goldberg EL. (1984). An epidemiological study of Hirschsprung's disease.Int J Epidemiol. 13(4): 479-485.
5. Gonzalo DH, Plesec T. (2013). Hirschsprung disease and use of calretinin ininadequaterectalsuctionbiopsies.ArchPatholLabMed. 137:1099-1102.
6. Hadeel A. Yasseen. (2015). Toluidine Blue Stain and Crystal Violet StainVersus H&E Stain in the Diagnosis of Hirschsprung'sDisease : A Studyin Sulaimani Cityin Kurdistan/Iraq. Annals of Pathology and Laboratory Medicine. 2(2): 1-8.
7. HiradfarM,SharifiN,KhajedalueeM,ZabolinejadN,TarazJS.(2012). CalretininimmunohistochemistryandaidinthediagnosisofHirschsprung's disease.IranJBasicMedSci. 15: 1053-1059.
8. Holland SK,Ramalingam P, Podolsky RH,Reid-Nicholson MD,LeeJR. (2011). Calretininimmunostainingasanadjunctinthediagnosisof Hirschsprungdisease.AnnDiagnPathol.15(5): 323-328.
9. Holschneider AM, W Meier-Ruge, B M Ure. (1994). Hirschsprung'sdiseaseandallieddisorders- Review. Eur J Pediatr Surg. 4(5): 260-266.
10. Ikeda K, Goto S. (1984). Diagnosis and treatment of Hirschsprung's disease inJapan.Ananalysisof1628patients.AnnSurg. 199(4): 400-405.
11. Jennifer Kessmann (2006). Hirschsprungs Disease: Diagnosis andManagement.AmericanFamilyPhysician.74(8): 1319-1322.
12. KacarAyper, Ata TurkerArikok, MujdemNurAzili, GunayEkberl. (2012). Calretininimmunehistochemistry in Hirschsprung's disease: An adjunct toformalin-baseddiagnosis. TurkJGastroenterol.23(3):226-233.
13. LavanyaKannaiyan,SujaniMadabhushi,RamaniMalleboyina, Narendra Kumar Are, K. Ramesh Reddy, BhuvaneshwarRao. (2013). Calretininimmunohistochemistry:Anewcost-effectiveandeasymethodfordiagnosisofHirschsprung's disease. JournalofIndian Association ofPediatricSurgeons.18(2): 66-68.
14. LuisDe la Torre, Karla Santos. (2012). Hirschsprungdisease.Evaluation of calretinin and S-100 as ancillary methods for the diagnosisofaganglionosis in rectal biopsies.ActaPediatricade Mex. 33(5): 246-251.
15. MałdykJ, Rybczyñska J, Piotrowski D,KozielskiR. (2014). Evaluationofcalretinin immunohistochemistry as an additional tool in confirming thediagnosisofHirschsprungdisease.PolJPathol. 65: 34-39.
16. Marielle Rodrigues Martinsa, Carlos Henrique Marques dos Santosa,GustavoRibeiroFalcaoa. (2015). LatediagnosisofHirschsprung'sdisease.JColoproctology. 5(3): 178–181.
17. Mukhopadhyay B, Mukhopadhyay M, Mondal KC, Sengupta M, PaulA. (2015). Hirschsprung'sDiseaseinNeonateswithSpecialReferencetoCalretininImmunohistochemistry.JClinDiagn Res. 9: 6-9.
18. PensabeneL,YoussefNN,GriffithsJM,DiLorenzoC. (2003). Colonic manometryinchildrenwithdefecatorydisorders: roleindiagnosisandmanagement.AmJGastroenterol. 98(5): 1052-1057.
19. Spouge D, Baird PA. (1985).Hirschsprung disease in a large birth cohort.Teratology. 32(2): 171-177.
20. TiffinME,ChandlerLR,FaberHK.(1940).Localizedabsenceofganglion cells of themyenteric plexus in congenitalmegacolon. Am JDisChild.59: 1071-1082.
21. Vincent Guinard-Samuel, Arnaud Bonnard, Pascal De Lagausie, PascalePhilippe-Chomette,CorineAlberti,AlaaElGhoneimi,MichelPeuchmaur, DominiqueBerrebi-Binczak. (2009). Calretininimmunohistochemistry:asimpleandefficienttooltodiagnoseHirschsprung disease. ModernPathology.22(10):1379-1384.
22. WhitehouseF,KernohanJ. (1948). Myentericplexusesincongenitalmegacolon studyof11cases.ArchIntMed. 82(1): 75-111.
23. ZuikovaV,FranckevicaI,StrumfaI,MelderisI. (2015).ImmunohistochemicaldiagnosisofHirschsprung'sdiseaseandallieddisorders.ActaChirurgicaLatviensis.15: 50-57.

ISSN (O): 2687-5365; ISSN (P): 2753-6556