COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY IN HIRSCHSPRUNG DISEASE

Dissertation submitted in

partial fulfilment of the requirements for the degree of

M.D. PATHOLOGY

BRANCH- III

INSTITUTE OF PATHOLOGY

MADRAS MEDICAL COLLEGE

CHENNAI- 600003

THE TAMILNADU DR M.G.R. MEDICAL UNIVERSITY

CHENNAI

MAY 2019

CERTIFICATE

This is to certify that this Dissertation entitled **"COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNO HISTOCHEMISTRY IN HIRSCHSPRUNG DISEASE"** is the bonafide original work of **DR.M.S.MUTHU PRABHA**, in partial fulfillment of the requirement for M.D., (Branch III) in Pathology examination of the Tamilnadu Dr.M.G.R Medical University to be held in May 2019.

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DECLARATION

I, **Dr.M.S.MUTHU PRABHA,** solemnly declare that the dissertation entitled **"COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY IN HIRSCHSPRUNG DISEASE"** is the bonafide work done by me at the Institute Of Pathology, Madras Medical College under the expert guidance and supervision of **Prof.Dr.Geetha Devadas,** M.D., DCP, Professor of Pathology and **Dr.R.NARMADHA,** M.D., Assistant professor of Pathology, Institute Of Pathology, Madras Medical College. The dissertation is submitted to the Tamilnadu Dr.M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch III) in Pathology.

Place : Chennai

Date : **DR.M.S.MUTHU PRABHA**

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Last but not the least, I am grateful to my family members and friends for their constant support and belief in me.

INSTITUTIONAL ETHICS COMMITTEE
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CERTIFICATE OF APPROVAL

To
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Dear Dr.M.S.Muthu Prabha,

The Institutional Ethics Committee has considered your request and approved your study titled **"COMPARATIVE STUDY OF TOLUDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY IN HIPSCHSPRUNG DISEASE" - NO.20122017**

The following members of Ethics Committee were present in the meeting hold on **05.12.2017** conducted at Madras Medical College, Chennai 3

1. Prof.P.V.Jayashankar :Chairperson
2. Prof.R.Narayana Babu,MD.,DCH., Dean,MMC,Ch-3 : Deputy Chairperson
3. Prof.Sudha Seshayyan,MD., Vice Principal,MMC,Ch-3 : Member Secretary
4. Prof.N.Gopalakrishnan,MD,Director,Inst.of Nephrology,MMC,Ch : Member
5. Prof.S.Mayilvahanan,MD,Director,Inst. of Int.Med,MMC, Ch-3 : Member
6. Prof.A.Pandiya Raj,Director, Inst. of Gen.Surgery,MMC : Member
7. Prof.Shanthy Gunasingh, Director, Inst.of Social Obstetrics,KGH : Member
8. Prof.Rema Chandramohan,Prof.of Paediatrics,ICH,Chennai : Member
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11. Prof.Bharathi Vidya Jayanthi,Director, Inst. of Pathology,MMC,Ch-3: Member
12. Thiru S.Govindasamy, BA.,BL,High Court,Chennai : Lawyer
13. Tmt.Arnold Saulina, MA.,MSW., :Social Scientist
14. Thiru K.Ranjith, Ch- 91 : Lay Person

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary – Ethics Committee

PLAGIARISM CERIFICATE

This is to certify that this dissertation work titled **"COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY IN HIRSCHSPRUNG DISEASE"** of the candidate **Dr.M.S.MUTHU PRABHA** with registration Number **201613005** for the award of **M.D PATHOLOGY** (Branch-III). I personally verified the urkund.com website for the purpose of Plagiarism Check. I found that the uploaded thesis file contains from introductionto conclusion and result shows **3 percentage** of plagiarism in the dissertation.

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Instances where selected sources appear:

10

ABBREVIATIONS

HD	:	Hirschsprung Disease
HSCR	:	Hirschsprung Disease
H & E	:	Hematoxylin And Eosin
HPE	:	Histopathological Examination
ENS	:	Enteric Nervous System
CNS	:	Central Nervous System
PNS	:	Peripheral Nervous System
ANS	:	Autonomic Nervous System
NCC	:	Neural Crest Cells
ENCC	:	Enteric Neural Crest Cells
EDNRB	:	Endothelin Receptor Type B
ET	:	Endothelin
GDNF	:	Glial Cell- Derived Neurotrophic Factor
IHC	:	Immunohistochemistry
AChE	:	Acetylcholiesterase Enzyme
ECM	:	Extra Cellular Matrix

CONTENTS

INFORMATION SHEET

We are conducting **"COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY IN HIRSCHSPRUNG DISEASE"** among patients **attending INSTITUTE OF CHILD HEALTH AND HOSPITAL FOR CHILDREN,** Chennai and for that your specimen may be valuable to us.

- The purpose of this study is to evaluate special stain are equally having similar results as that of immunohistochemistry.
- We are selecting certain cases and if your specimen is found eligible, we may be using your specimen to perform extra tests and special studies which in any way do not affect your final report or management.
- The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in this study is voluntary. You are free to decide whether to participate inthis study or to withdraw at any time; your decision will not result in any loss ofbenefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of investigator Signature of participant

Date:

INFORMED CONSENT FORM

Title of the study: **"COMPARATIVE STUDY OF TOLUIDINE BLUE SPECIAL STAIN AND IMMUNOHISTO CHEMISTRY IN HIRSCHSPRUNG DISEASE"**

Name of the Participant :

Name of the Principal(Co-Investigator) :

Name of the Institution : **INSTITUTE OF CHILD HEALTH AND HOSPITAL FOR CHILDREN**, Madras Medical College.

Name and address of the sponsor / agency (ies) (if any) :

Documentation of the informed consent

I _____ have read the information in this form (or it has been read tome). Iwas free to ask any questions and they have been answered. I am over 18 years of age and, exercisingmy free power of choice, hereby give my consent to be included as a participant in **"COMPARATIVE STUDY OF TOLUDINE BLUE SPECIAL STAIN AND IMMUNOHISTOCHEMISTRY IN HIRSCHSPRUNG DISEASE"**

1. I have read and understood this consent form and the information provided to me.
2. I have had the consent document explained to me.
3. I have been explained about the nature of the study in which the submitted rectal biopsy will be subjected to immunocytochemistry and special stain examination.
4. I have been explained about my rights and responsibilities by the investigator. I have the right to withdraw from the study at any time.
5. I have been informed the investigator of all the treatments I am taking or have taken in the past _____ months including any native (alternative) treatment.
6. I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Govt. agencies, and IEC. I understand that they are publicly presented.
7. I have understood that my identity will be kept confidential if my data are publicly presented
8. I have had my questions answered to my satisfaction.
9. I have decided to be in the research study.

I am aware that if I have any question during this study, I should contact the investigator. By signing this consent form I attest that the information given in this document has been clearly explained to me and understood by me, I will be given a copy of this consent document.

For adult participants:

Name and signature / thumb impression of the participant (or legal representative if participant

incompetent)

Name _____ Signature_____
Date_____

Name and Signature of impartial witness (required for illiterate patients):

Name _____ Signature_____
Date_____

Address and contact number of the impartial witness:

Name and Signature of the investigator or his representative obtaining consent:

Name _____ Signature_____
Date_____

ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சி தலைப்பு : ஹிர்ஸ்பிரங் (Hirschsprung) நோயில் டொலிடின் ப்ளூ கறை மற்றும் இம்மினோஹிஸ்டோகெமிஸ்ட்ரி ஆகிய இரண்டின் ஒப்பீட்டு ஆய்வு.

சென்னை மருத்துவக் கல்லூரி நோய்குறியியல் துறையில் மரு. மு.செ.முத்துப்பிரபா அவர்கள் மேற்கொள்ளும் இந்த ஆய்வில் பங்குகொள்ள ..... ஆகிய நான் முழு மனதுடன் சம்மதிக்கிறேன்.

எனக்கு விளக்கப்பட்ட விஷயங்களை நான் புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்ப்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும் அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் நான் புரிந்து கொண்டேன்.

நான் மலக்குடலில் ஏற்படும் ஹிர்ஸ்பிரங் (Hirschsprung) நோயினை குறித்த இந்த ஆராய்ச்சியின் விவரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக் கொண்டேன்.

நான் என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக் கொள்ள சம்மதிக்கிறேன்.

எனக்கு அறுவை சிகிச்சை செய்யப்பட்டு நோய்க்குறியியல் துறையில் திசுப் பரிசோதனைக்கு பயன்படுத்தப்படும் திசுவினை மெழுகுக்கட்டிகளை வைத்து ஆராய்ச்சி மற்றும் சிறப்புப் பரிசோதனை செய்து கொள்ள சம்மதம் தெரிவிக்கிறேன்.

பங்கேற்பாளர் கையொப்பம்..... இடம் :..... தேதி :.....

பங்கேற்பாளர் பெயர் மற்றும் விலாசம் .....

ஆராய்ச்சியாளர் கையொப்பம்..... இடம் :..... தேதி :.....

ஆராய்ச்சி தகவல் தாள்

ஆராய்ச்சி தலைப்பு : **ஹிர்ஸ்பிரங் (Hirschsprung) நோயில் டொலிடின் ப்ளூ கறை மற்றும் இம்மினோஹிஸ்டோகெமிஸ்ட்ரி ஆகிய இரண்டின் ஒப்பீட்டு ஆய்வு.**

ஆய்வாளர் : மரு. மு.செ. முத்துப்பிரபா,
இரண்டாம் ஆண்டு,
நோய்குறியியல் துறை,
சென்னை மருத்துவக் கல்லூரி,
சென்னை - 600003.

தங்களது மலக்குடலில் (அறுவை சிகிச்சை செய்யப்பட்ட திசு) இங்கு பெற்றுக்கொள்ளப்பட்டது.

இராஜீவ் காந்தி அரசு பொது மருத்துவமனைக்கு வரும் நோயாளிகளிடம் இருக்கும் மலக்குடலில் ஏற்படும் நோயினை பற்றி ஒரு ஆராய்ச்சி இங்கு நடைபெற்று வருகின்றது.

மலக்குடலில் ஏற்படும் ஹிர்ஸ்பிரங் (Hirschsprung) நோயினை டொலிடின் ப்ளூ கறை மற்றும் இம்மினோஹிஸ்டோகெமிஸ்ட்ரி ஆகிய இரண்டின் ஒப்பிடுதலே இந்த ஆய்வின் நோக்கமாகும்.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். இந்த ஆராய்ச்சியில் உங்களுடைய திசுக்கள் எடுத்து சில சிறப்புப் பரிசோதனைக்கு உட்படுத்தி அதன் தகவல்களை ஆராய்வோம். அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்குள்ளாகாது என்பதையும் தெரிவித்துக் கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துகளை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிட மாட்டோம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது. மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியில் இருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த சிறப்புப் பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆய்வை பற்றிய சந்தேகங்களுக்கு தொடர்பு கொள்ள வேண்டியவர் : மரு. மு.செ.முத்துப்பிரபா, செல் : 9841266349

பங்கேற்பாளர் கையொப்பம்..... இடம் :..... தேதி :.....

பங்கேற்பாளர் பெயர் மற்றும் விலாசம் .....

ஆராய்ச்சியாளர் கையொப்பம்..... இடம் :..... தேதி :.....

INTRODUCTION

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[1,2.] 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.[3,4] The condition leads to loss of tonic neural inhibition ,persistent contraction of the affected segment and subsequent colonic obstruction. The condition usually is due to defective craniocaudal migration of vagal neural crest cells (the progenitors of ganglion cells) between gestational weeks 5 and 12[4].Interruption of cranio-caudal migration of neural crest cells gives the explanation for distal aganglionosis in Hirschsprung disease. The aganglionic segment in Hirschprung disease begins at the anal sphincter and extends proximally[5]. The diagnosis of HD 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 [6,7].In addition , there might be an increase in non-myelinated cholinergic nerve fibers in the sub-mucosa and among muscle layers (neural hyperplasia), which helps in diagnostic confirmation[8]. Calretinin stains ganglion cells and calretinin positivity rules out disease[9,10,11,12,13]. S-100 immunostains can highlight the presence of hypertrophic submucosal nerve fibers[13,14].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[15].

Surgery is the treatment of choice for Hirschsprung disease. It also prevents further complication of enterocolitis[16].

This study is to assess the efficacy of toluidine blue staining in the detection of ganglion cells and confirm with immunohistochemistry. In centres were immunohistochemistry is not readily available,toluidine blue can be used as a cost effective substitute to immunohistochemistry

AIMS & OBJECTIVES

AIMS AND OBJECTIVES

To evaluate the diagnostic efficacy of toluidine blue special stain in diagnosing Hirschsprung disease when compared to immunohistochemistry – Calretinin and S-100

REVIEW OF LITERATURE

REVIEW OF LITERATURE

EPIDEMIOLOGY

The incidence of HSCR varies significantly among different geographic distribution- 1.5, 2.1, and 2.8 per 10,000 live births in Caucasians, African-Americans, and Asians, respectively[17]. HSCR occurs isolated (non-syndromic) in about 70% of cases, whereas it is associated with other congenital malformations (including the syndromic form) in 18% of cases and with a chromosomal abnormality in 12%[18]. The congenital malformations commonly reported along with HSCR are cardiac (5%), renal (4.4%), genital (2-3%). Isolated HSCR cases appears to be a multifactorial defect with sex modified penetrance and variable expression in the length of the aganglionic segment. Familial forms of HSCR represent 6-15% of the cases[19]. There is a preponderance towards males and the male to female sex ratio is 4:1 and also more commonly associated with first born male child . A large number of associated conditions have been reported with disease, including Down syndrome, cardiovascular malformations, neurofibromatosis, Waardenburg syndrome, Laurence-Monn-Bardet-Biedl syndrome, Ondine's curse (Haddad syndrome), multiple endocrine neoplasia, neuroblastoma, total colonic agenesis, and imperforate anus, many of which belong to the category of neural crest disorders ("neurocristopathies")[20,21]. Among all the associated conditions Hirschsprung disease most commonly present in Down syndrome. Enterocolitis is more common in Hirschsprung disease . Enterocolitis presents either along the disease or as a post -operative complication[21].

Embryology

The enteric nervous system is also called as the second brain. The enteric nervous system (ENS) is the largest and most complex division of the peripheral and autonomic nervous systems (PNS and ANS) in vertebrates. It contains numerous different types of neurons in comparison to that of the spinal cord. The ENS is organised into an interconnected network of neurons and glial cells that are grouped into ganglia located in two major plexuses- the myenteric (Auerbach's) plexus and the submucosal (Meissner's) plexus. ENS components form an integrated circuitry that controls motility of the intestine, exchange of fluids across the mucosal surface, blood flow and secretion of gut hormones. Although the gut also receives extrinsic parasympathetic and sympathetic innervation, the intrinsic neuronal circuits of the ENS are able to generate reflex gut contractile activity independent from any CNS intervention, setting the ENS apart from other components of the ANS[22]

The neural crest origin of the ENS was first established by Yntema and Hammond who showed that upon ablation of the vagal (hindbrain)region of the neural crest in avian embryos, enteric ganglia failed to form along the length of the gastrointestinal tract[23.] These results were subsequently confirmed in chick embryos by Le Douarin. This was studied by transplanting quail cells in chick embryos.

Fate mapping of the transplanted quail cells revealed a dual neural crest origin for the ENS in which neural crest cells (NCC) emigrating from the vagal region adjacent to somites 1–7 colonised the entire length of the gut forming

the majority of the ENS, whereas NCC arising caudal to the 28th somite level (sacral NCC) contributed a smaller number of cells to the post-umbilical gut only[24]

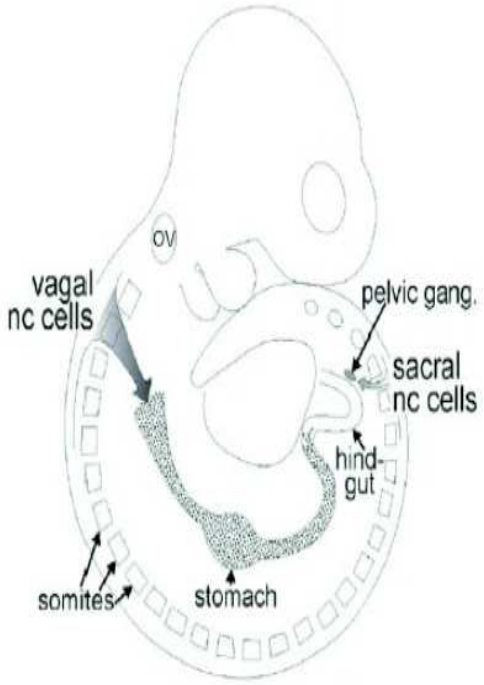


FIG-1 NEURAL CREST CELL[22]

Vagal NCC emerge out of the neural tube around embryonic day 8.5(E8.5) in the mouse and migrate ventro-medially arriving the foregut at E9–E9.5 [25.] In foregut NCC are termed as enteric neural crest cells (ENCC), since it contains ENS-restricted progenitor cells that migrate rostrocaudallyto colonise the entire length of the developing gut. The process takes approximately 5 days (by E14.5–E15.5).Sacral NCC emerges outfrom the neural tube at E9–E9.5, migrate ventrally to form extrinsic pelvic ganglia adjacent to the hindgut, then migrate from there into the gut to give rise to enteric neurons and glia[26]. Sacral NCC enters into the hindgut only after the

arrival of their vagal counterparts[26,27] . Vagal and sacral NCC express the transcription factor Sox10 from the onset of migration till they reach gut[25,28]. Vagal NCC also express EDNRB and p75 in the migratory phase[25,29]. They also have the expression of the receptor tyrosine kinase RET [25].Recent studies says that both vagal and sacral NCC has similar expression[25,30]

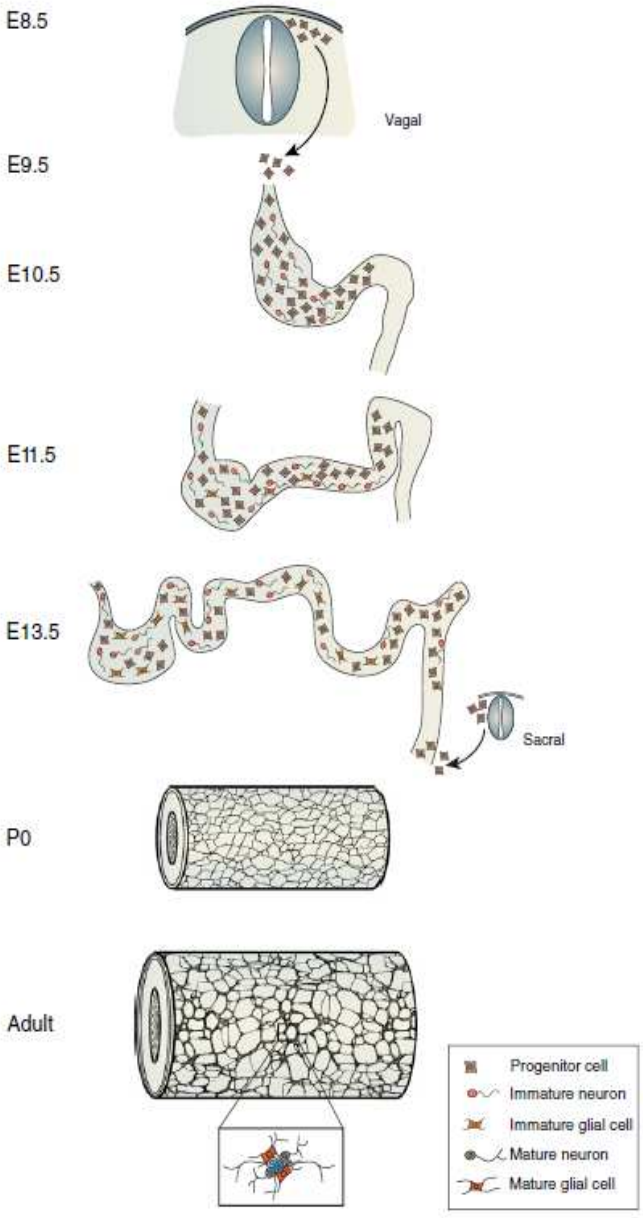


FIG 2-COLONISATION OF NEURAL CREST CELL IN GIT [22]

Molecular mechanisms controlling ENCC colonisation of the gut.

ENS development is a complex and asynchronous process. There are certain molecular mechanisms that controls the colonisation of the gut.

1.Cell population

2. Proliferation and survival

3.Cell migration

CELL POPULATION

The population size of pre-enteric NCC plays an important role in determining the extent of gut colonisation[23,31,32]. Recently it showed that minimal number is necessary for the complete rostrocaudal colonisation of the gastrointestinal tract[33]. Recent frontal expansion model showed that there is an unidirectional colonisation of the gut accomplished by cells which are in the front are acting as a proliferative source to generate enough motile ENCC to invade previously unoccupied regions, while those behind the front are essentially non proliferative and do not participate directly in the invasion of unoccupied tissues [34].

PROLIFERATION AND SURVIVAL

Three major groups of molecules have been implicated to inthe control of ENCC proliferation and survival during ENS development. They are RET-GDNF, Endothelin-3(ET3)-EDNRB and transcriptionfactors such as Sox10 and Phox2b.

RET

RET is a transmembrane tyrosine kinase receptor for the glial cell line-derived neurotrophic factor (GDNF) family of ligands including GDNF, neurturin, artemin and persephin [35]. The ligands are able to activate RET by binding to a preferred high-affinity glycosylphosphatidylinositol (GPI)-anchored co-receptor GFRα (GDNF family receptor α-component)[36,37]. RET expression is seen in migratory ENCC and later found exclusively in the neuronal population[38,39,40]. The mutation in the RET or GDNF genes leads to total intestinal aganglionosis, because the ENCC fail to colonise the gastrointestinal tract beyond the rostral stomach .This was confirmed by a mouse model[41,42,43,44,45,46].

EDNRB

EDNRB is a G protein-coupled receptor for endothelin-1 (ET-1), ET-2 and ET-3 [47]. ET-3 is expressed by the gut mesenchyme [48,49] . The mutations of the EDNRB or Et-3 locus lead to the absence of enteric neurons from the terminal region of the gut (colonic aganglionosis) [50,51,52]. Absence of ET-3 leads to migratory delay in colonisation of the gut [48], associated with a decreased pool of proliferative progenitors and also with premature neuronal differentiation at the front of migration [53].

Sox10

Sox10 a member of the SRY-box containing (Sox) family of transcription factis expressed in NCC[54.]Sox10 levels are necessary for maintenance of the ENS progenitor pool [55].

Phox2b

Phox2b a paired-homeodomain transcription factor which iswidely expressed NCC [56] but their expression occurs after the expression of Sox10. In the absence of Phox2b, vagal NCC enter the foregut but it fails to express markers such as RET, Mash1 and TH . These suggests a role Phox2b in controlling the ENCC survival by direct regulation of RET expression[57].

CELL MIGRATION

There are various processes involving enteric neural crest cells in migrating through the gut mesenchyme. RET–GDNF signalling pathway seems to play a key role in retaining NCC within the gut mesenchyme, and also in inducing their rostro-caudal migration [58,59].ET-3 might be able to negate GDNF attraction and enhance the migratory properties of ENCCs within the caecal region, allowing the cells to progress further instead of stalling[60]. Another factor which influences cell migration is the cell interaction and cell adhesion to the extracellular matrix. Altered expression of ECM and any defect in adhesion leads to incomplete colonisation of the gastrointestinal tract[61,62]. ET-3 mutations alters the expression of laminin in the gut mesoderm and, along with

the cell-autonomous effects of the mutation, leads to terminal aganglionosis[63,64,65].

All these three molecular mechanisms are necessary for the complete development of ENCC and colonisation of the GIT .Any mutation or defect in this mechanism would lead to colonic aganglionosis.

ANATOMY

The sigmoid colon begins where the descending colon passes in front of the pelvic brim and below it continues with rectum at the level of S3.

It measures upto 15 to 45 cm. Sigmoid colon is a s-shaped shaped structure.

Sigmoid colon is an intraperitoneal structure. Sigmoid colon is mobile upto a certain extent. It hangs down into pelvic cavity in the form of loop. It is attached to the posterior wall of the pelvis by a fan shaped fold of peritoneum known as sigmoid mesocolon.

The rectum forms the distal 8–15 cm of extraperitoneal large bowel that lies within the pelvis and ends at the anal canal. The rectosigmoid junction is found in between the sigmoid and rectum which is 15 to 17 cm from the anal verge. The rectum gets the blood supply from three main arteries-superior rectal artery, middle rectal artery and inferior rectal artery[66].

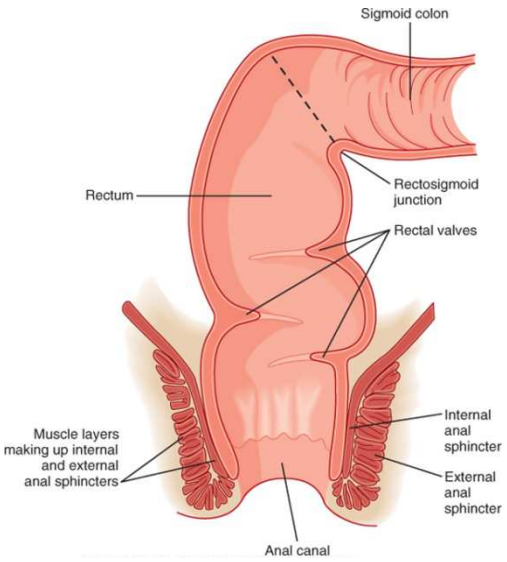


Fig 3- anatomy of sigmoid colon and rectum[66]

Histology

The gastrointestinal tract has functional four layers.

Mucosa- comprises of three components the epithelium,Lamina propria and a thin smooth muscle layer muscularis mucosa

Submucosa

Muscularis propria

Adventitia

COLON HISTOLOGY

Mucosa - consists of two types of cells, absorptive and mucus secreting goblet cells. The cells are arranged in closely packed straight tubular glands or crypts. The glands or crypts extend down to lie on the muscularis mucosae. The muscularis mucosae keeps the mucosal surface and glands, helps in expelling the secretions from the deep glandular crypts, prevents clogging and also enhances contact between epithelium and luminal contents for absorption.

Lamina propria is the space between glands and it contains numerous blood vessels.

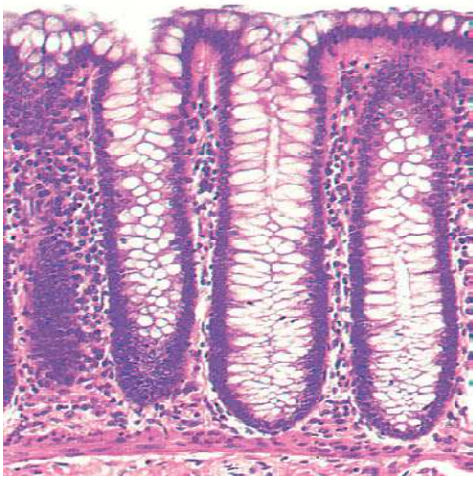


Fig 4. MUCOSA OF COLON[67]

Submucosa is a layer with loose connective tissue that supports mucosa and contains larger blood vessels, lymphatics and nerves supplying mucosa. Submucosa composed of tiny parasympathetic ganglia. Parasympathetic ganglia which forms the submucosal (Meissner) plexus from which postganglionic fibres supply the muscularis mucosae.

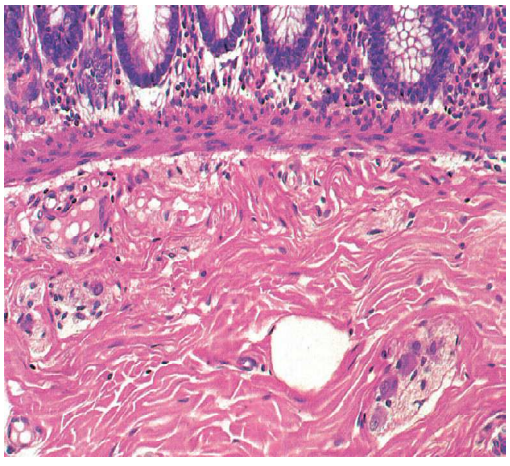


FIG- 5 Parasympathetic ganglia in submucosa[67]

Muscularis propria is thick walled and it consist of inner circular and outer longitudinal layer. Between the both muscular layers, there are clumps of pale-stained Parasympathetic ganglion cells of the **myenteric (Auerbach) plexus**. The two layers of the muscularis propria undergo synchronised contractions that pass in **peristaltic waves** down the tract, propelling the contents distally. Actually the peristalsis is initiated by the pacemaker cells known as , the **interstitial cells of Cajal**, but the level of activity is controlled by the autonomic nervous system. This is done by means of locally produced gastrointestinal tract hormones and by other environmental factors. Parasympathetic activity enhances peristalsis while sympathetic activity slows gut motility.

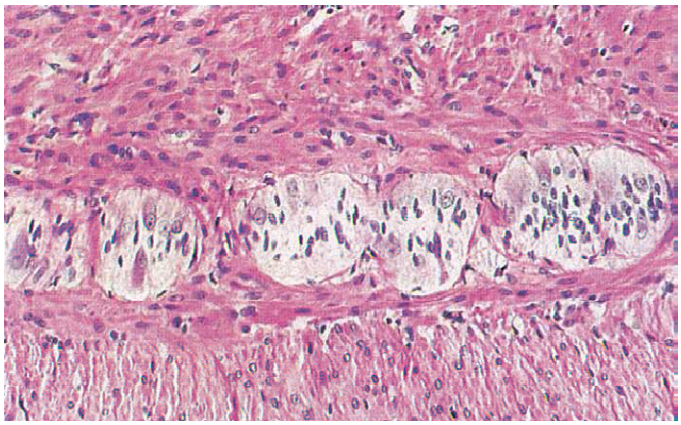


FIG 6 - Parasympathetic ganglia in muscularis propria[67]

Adventitia is the outermost loose layer and it has major vessels,nerves and also the adipose tissue [67].

CLINICAL FEATURES

About 80% of patients present in first few months of life.

Nearly 90% of patients are full term babies with normal weight. They present with failure to pass meconium in first 24 hours of life.

Symptoms of Hirschsprung disease in infants

Failure to pass meconium in first 24 hours of life

Bilious vomiting

Enterocolitis associated diarrhea

Decreased bowel movements

Jaundice

Poor feeding

Progressive abdominal distension

Tight anal sphincter with an empty rectum

Symptoms of Hirschsprung disease in older children

Failure to thrive

Fecal impaction

Constipation

Malnutrition

Abdominal distension

Absence of overflow incontinence or soiling

Symptoms will resolve with enema,laxatives but it will recur[68,69].

TYPES OF HIRSCHSPRUNG DISEASE

There are five types of Hirschsprung disease

Short segment, Long segment, Ultra short segment, Total colonic aganglionosis, Total intestinal aganglionosis[70,71] .

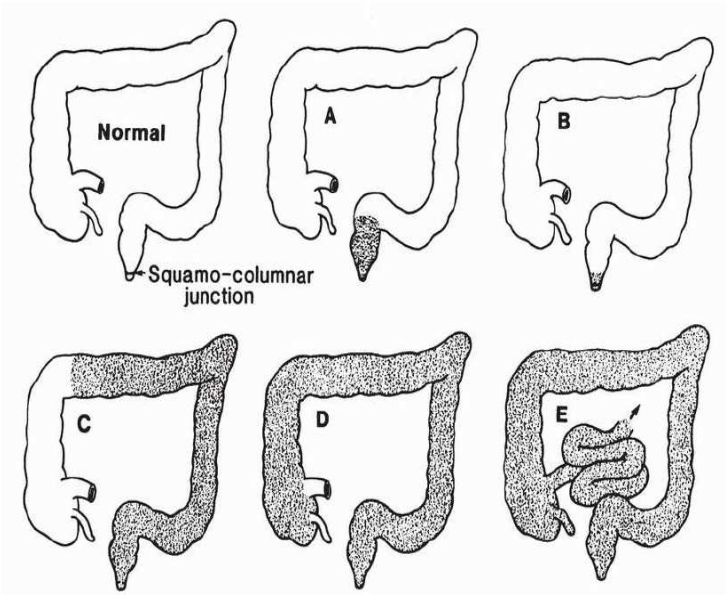


FIG 7 TYPES OF DISEASE[72]

A- Short segment- this type is the most common one with 80% incidence , and this type is most common in males and it is restricted upto the rectosigmoid colon ,

B- Ultra short segment-is rare affecting distal rectum near anal sphincter.

C- Long segment – 10- 15%disease involvement upto hepatic flexure.

D- Total colonic aganglionosis- 5% ,involvement of entire colon.

E- Total intestinal aganglionosis-, involving the entire colon and distal small intestine.

INVESTIGATIONS

Careful clinical examination is required. For establishing the diagnosis, a number of diagnostic studies should be performed.

Abdominal X Ray

Plain abdominal radiographs may shows distended bowel loops with absent air in the rectum[73,74].

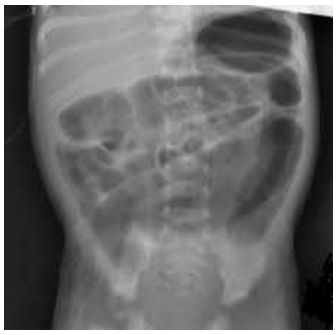


Fig 8-distended bowel loops with air absence in rectum[74]

Contrast Enema

Basically barium enema radiograph of colon will appear normal till 3 months of life though there would be a total aganglionosis. It starts manifesting after the bowel gets dilated.

Contrast enema radiograph will show a narrowed distal colon which is the affected part with proximal dilation which is the a classic finding of HSCR. Another positive finding is the retention of barium for longer than 24 hours after the procedure has been performed [75,76].

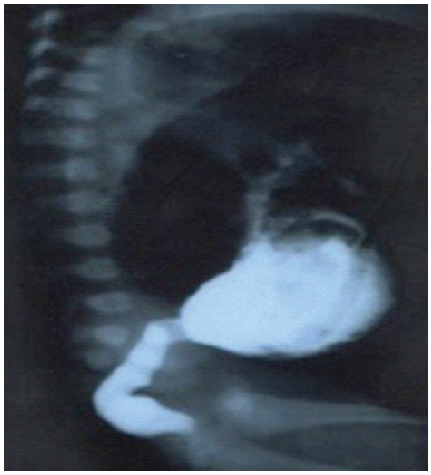


Fig 9-showing narrow rectosigmoid with dilated bowel[77]

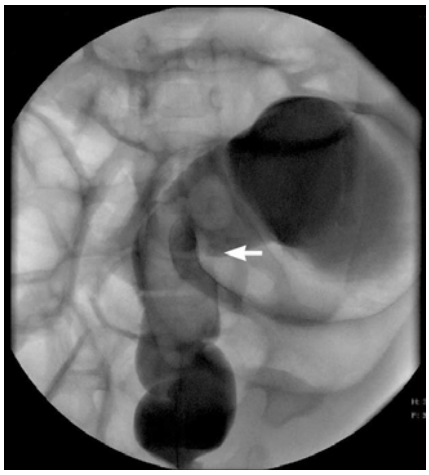


Fig 10 – Shows the transtition zone

Anal manometry

The internal anal sphincter provides most of the support in the anal canal, and rectal distention causes reflex relaxation of the sphincter. This is known as the rectosphincteric reflex. This can be artificially induced by an anorectal manometer. This is an atraumatic technique, by using a balloon device, which induces rectal distention. The Rectosphincteric reflex is absent in patients with Hirschsprung disease, but is present in patients with chronic constipation of other etiologies[78]. Anal manometry shows balloon distention of the rectum which demonstrates the absence of internal anal sphincter relaxation upon rectal distention[79.]

Rectal biopsy

This is the gold standard method for diagnosing Hirschsprung disease[,80,81]. This is the confirmatory test but with some disadvantage namely interobserver variation can be present.

There are certain indications for rectal biopsy in children less than 6 months of age and they are

(a) delayed meconium passage;

(b) low intestinal obstruction of unknown cause;

(c) severe constipation;

(d) chronic abdominal distention and

(e) failure to thrive[82,83,84].

Biopsy pre requisites are

i)Biopsy site should be 3mm diameter with sub mucosa representing one third of thickness.

ii) Biopsy should be taken at a point 2 cm above the anal valve in infants and 3cm in older children,

iii)The presence of squamous or transitional epithelium in a specimen indicates that the level of the biopsy is too low.[72]

Types of rectal biopsy

- Rectal suction biopsy
- Open rectal biopsy
- Endo rectal pull through

After receiving biopsy it is the duty of the pathologist to concentrate on embeding, because it plays a vital role in identifying the ganglion cells.

Rectal suction biopsy

It is the simple, safe, easy method without general anaesthesia.

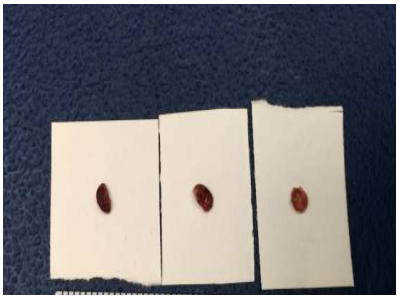


Fig 11- Place on cardboard mucosa side up

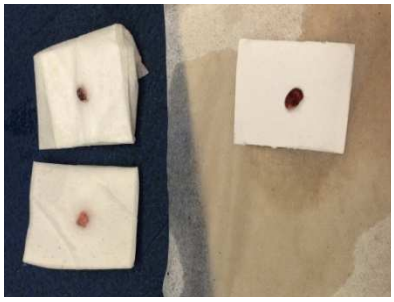


Fig 12- Wrap cardboard in lens paper

Fig 13- Place wrapped cardboard in biopsy bag and place in cassette

- Specimen should be placed in one piece per cassette. (usually 2-3 pieces are received)
- <u>Orientation</u>

    1) The tissue should be placed on a piece of cardboard mucosa side up.
    2) Wrap the cardboard in lens paper.
    3) Place the lens paper in a biopsy bag.
    4) Place the biopsy bag in the cassette.

Open rectal biopsy

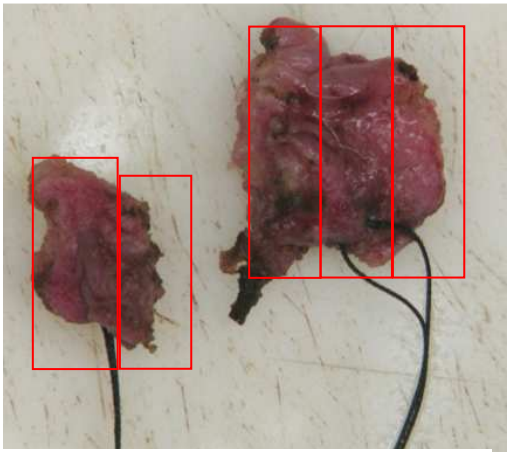


Fig 14- open rectal biopsy

- First orientation of the specimen is done by inking
- Sections should be cut perpendicular
- Put each section in separate cassette and orientation on the edge

Endorectal Pull-Through

The specimen consists of segment of colon- full thickness bowel proximally and mucosal/Submucosal tube distally**.**

1. Specimen should be oriented.
2. Specimen is cut open and pinned out or fixed flat for ideal sectioning**.**
3. Photograph of both serosal and mucosal surfaces is taken. Measure the length and internal circumference of the segment received describe the serosal surface.

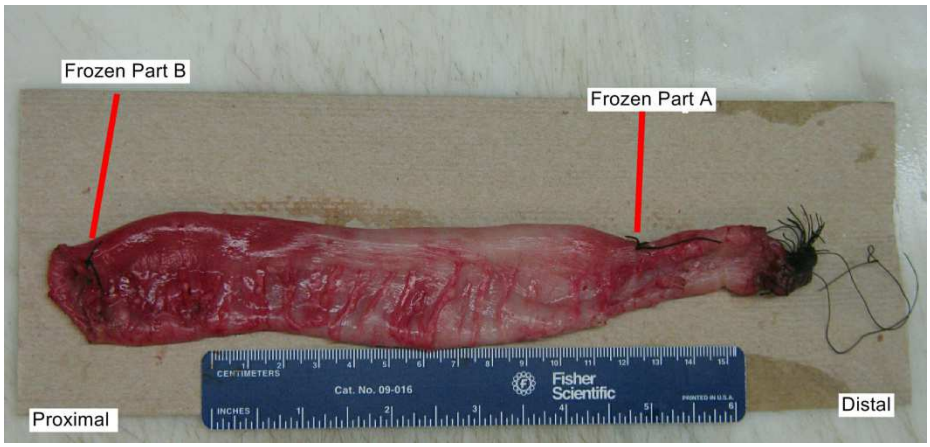


Fig 15- full thickness colon

6. The mucosal surface is evaluated .
7. Proximal shave margin is taken , submitting the entire margin on edge
8. One complete longitudinal section from proximal to distal margin is to be submitted, including prior biopsy sites.
9. Put one piece per cassette and ink the proximal end of each piece the same color to maintain orientation[85].

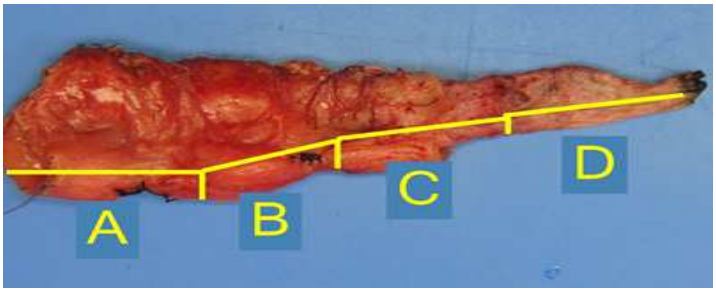


Fig 16- SECTIONS TO BE TAKEN IN FULL THICKNESS COLON

Histopathology

Submucosa is evaluated for ganglion cells. The pathologist should comment about the absence of ganglion cells.For this adequate sampling and lot of sectioning > 75 should be done.The diagnosis of Hirschprung disease is done by the absence of ganglion cells in the Myentric and Ayurbachs plexus . The presence of hypertrophic nerve fibres >40 mm is an added positive finding which should be searched[86]

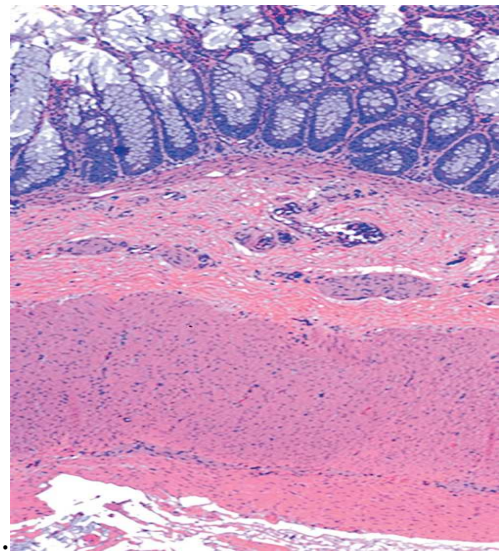


FIG -17 ABSENCE OF GANGLION CELLS AND HYPERTROPHIC NERVE TRUNKS

ROLE OF IMMUNOHISTOCHEMISTRY (IHC)

Albert Coons et al performed IHC by using labelled antibodies with fluorescent isocyanate in the year 1941. In the year 1966, Nakene and Pierce introduced the indirect labelling technique. The procedure in this was the unlabelled antibody followed by second antibody or substrate. Then various other methods were then introduced.

Antigen retrival

It is the procedure done to unmask the antigenic determinants in a formalin fixed tissue section,for which following methods are used.

1.Proteolytic enzyme digestion.

2. Microwave antigen retrieval.

3. Pressure cooker antigen retrieval.

4. Microwave and trypsin antigen retrieval.

Microwave antigen retrival is the most common method used widely as it allows rapid and uniform heating to the tissues. Pressure cooker retrival has an advantage of less time consumption and using large amount slides for antigen retrival.

Disadvantages of pretreatment

1.Sections should not be allowed to dry after antigen retrival.

2.Nuclear features would be destroyed in a poorly fixed smear

3.Fibers and fatty tissue would wash off during the procedure

4.All the antigens will not be retrived and so altered staining pattern could be seen.

DETECTION SYSTEMS

In the procedure when specific antibodies are added to the antigen,the next step is to visualize the antigen antibody reaction,for which there are two methods.

1.Direct method

2.Indirect method.

Direct method – primary antibody is conjugated with labels such as fluoro-chrome, horse radish peroxidase and alkaline phosphatase

Indirect method is a two step method in which labelled secondary antibody reacts with primary antibody bound to specific antigen.

ROLE OF IHC

Immunohistochemistry (IHC) comes to play a role when there is difficulty in identifying the ganglion cells. Sometimes ganglion cells of the neonate are immature and it would be difficult to recognize in routine H& E sections more commonly in the sub-mucosa. Endothelial cells and neuronal cells may also lead to a difficulty in diagnosing Hirschsprung disease. Immunohistochemistry will help in identifying the ganglion cells in such instances.

There are many IHC markers used for Hirschsprungdisease and they are Calretinin

Neuron-specific enolase (NSE),

RET oncoprotein,

Bcl-2,

Cathepsin D,

Protein gene product 9.5 (PGP9.5),

HuC/D,

Neu N - this is more sensitive and specific and it is the most preferred marker.

S-100 -highlights the presence of hypertrophic nerve bundles[72].

CALRETININ

Calretinin is a member of superfamily of calcium binding protein. It is abundantly expressed in central and peripheral neural tissue. Nerve cell bodies in both sub- mucosa and myenteric ganglia of the human gastrointestinal tract are immunopositive for calretinin[87.] It has been recently suggested that calretinin might be more accurate than acetylcholinesterase in detecting aganglionosis in a series of rectal biopsy[88.] Calretinin shows positivity in ganglion cells but calretinin immunostaining was always negative in nerve fibers, but in certain situation calretinin showed slight positive staining of some nerve fibers.

In short-segment HD, a slight calretinin positivity can be observed in some large nerve bundles and could indicate the beginning of a transitional zone. Calretinin is found to be highly sensitive marker to identify the presence of ganglion cells[88,89]. Calretinin IHC is accurate in detecting the presence or absence of ganglion cells and holds several advantages such as follows:

(1) It can be carried out on paraffin-embedded tissue sections;

(2) Staining pattern is simple;

(3) Binary pattern of interpretation (negative or positive);

(4) It is cost effective

It serves as a valuable cost-effective diagnostic aid in the places where Acetylcholinesterase enzyme histochemistry is not available as it is more costly and acetylcholinesterase demonstration requires frozen section[90].

Various studies have shown that calretinin is more sensitive and specific in identifying ganglion cells in colon of Hirschprung disease.

Barshack et al. summarized that aganglionic segments revealed absence of calretinin expression in ganglion cells and in the nerve fiber in HD, and conversely calretinin expression was positive in both ganglion cells and nerve fibers in ganglionic areas of HD and normal colon[89]

Zuikova et al. stated that calretinin immunohistochemical technique is less challenging and can be interpreted more easily than AChE. In this study, all HD cases showed negative expression of calretinin in small nerve fibers of

the lamina propria, muscularis mucosae, and submucosa, . On the other hand, all non HD cases (diagnosed histopathologically by the demonstration of ganglionic cells) showed positivity for calretinin[91].

Małdyk et al. reported a study in 2014, showing that the expression of calretinin was positive in all rectal biopsies with ganglionic cells while negative expression was noticed in all aganglionic segments, thus concluding that immunohistochemical staining of calretinin is a good adjunct to histopathology in the diagnosis of HD[92].

Lim et al. recorded results of the 27 patients with HD, and concluded that immunostaining with calretinin is a reliable ancillary technique in the investigation of HD[93].

Alexandrescu et al., published in 2013 that calretinin immunohistochemical test is a also a diagnostic method when used in combination along with histopathological examination, particularly in cases where sparse or immature ganglion cells in colonic submucosa are present[94] .

Hiradfar et al. investigated the expression of calretinin in colonic sections of HD and showed that sensitivity and specificity of this method for the diagnosis of HD in full thickness specimens of intestinal wall were 93.3% and 100%, respectively, with a positive predictive value of 100% and negative predictive value of 93.8%[95] .

Mukhopadhyay et al. published their report as sensitivity of calretinin immunohistochemistry for ganglion cells detection was 100% and that the

specificity was 97.44%, with positive and negative predictive value of 84.62% and 100%, respectively [96].

Kaçar et al. concluded that immunohistochemical testing of calretinin has high sensitivity and specificity for the diagnosis of HD[97].

Gonzalo et al. showed in their retrospective study that all patients of Hirschprung disease showed negative calretinin expression giving the conclusion that immunohistochemical testing of calretinin is quite supportive along with hematoxylin and eosin stained section in a clinically suspicious cases[98].

S 100

S100 Immunostaining stains intrinsic nerve fibers and negatively stained ganglion cells surrounded by positive Schwann cells in a normal colon of non Hirschsprung disease, while in Hirschsprung disease affected tissue, intense and prominent S100 staining is seen in hypertrophy of nerve fibers [98,99]. Hirschsprung disease cases harboured hypertrophic nerve bundles of ≥40 mm. This is an additional criteria for diagnosing Hirschsprung disease[14].

Robey et al. first introduced S-100 immunostaining as an additional criteria for diagnosing Hirschsprung disease in the year 1988[100].

Monforte-Munoz et al later found that 90% of their cases harboured nerve bundles of ≥40 mm. They concluded that S-100 immunostaining as a useful ancillary modality in cases without demonstrable ganglion cells [14,101].

SPECIAL STAIN

Special stain using acetylcholinesterase can be used as special stain. Histochemical demonstration of acetylcholinesterase positive cholinergic nerve fibers within the lamina propria provides a supportive evidence for the diagnosis of disease. This will not be present in normal individuals.The pit falls of acetylcholinesterase is that it needs to be done in frozen section and also it is costly[72].some other cheaper stains showing metachromasia can also be used .some such stains are Toluidine blue, Geimsa, Diff-Quick stain and creysl violet to identify ganglion cells.

Toluidine blue stain : (also known as tolonium chloride) it is a synthetic, acidophilic metachromatic dye which has an high affinity for nucleic acids, and it binds to nuclear material with a high DNA and RNA content, both in chromatin or Nissl substance[102] and selectively stains nucleus blue and cytoplasm light blue[103]. Other acidic tissue components such as sulfates, carboxylates, and phosphate radicals stained in shades of blue. Toluidine blue stains in mast cells show metachromatic violet (due to histamine and heparin metachromatic granules) [104].

Toluidine blue stains the cytoplasm of both mature and immature ganglion cells a rich ultramarine blue ,this was a study done by A.G.Weinberg[105.]

Canil et al who used Toluidine blue stain and found that Toluidine blue method is a better method to identify ganglion cells in frozen rectal biopsies.

This method provides faster and easier identification of ganglion cells than with H&E staining[106] .

M. K. Babu et al used H&E-and Tb and AChE-stained sections in their study to prove the thickness of nerve trunks in the submucosa by using the Leitz oculometer [107] .

Hadeel A. Yasseen et al said that Toluidine blue should to be used as the routine stain to highlight the ganglion cells in all suspected cases of Hirschsprung disease. Submucosal nerve bundle hypertrophy has to be assessed as an adjuvant histolological criterion [15].

Only limited studies were found in toluidine blue.

COMPLICATIONS OF HIRSCHSPRUNG DISEASE

Generally patients are treated for Hirschsprung disease and so do not have complications. However, constipation, fecal incontinence are less common[108]. Enterocolitis and colonic rupture are the most serious complications associated with the disease and are the most common causes of Hirschsprung related mortality

However, postoperative enterocolitis, is a grave complication that may develop rapidly which is due to the vascular compromise caused by distal colonic obstruction and superimposed bacterial infections. Enterocolitis later on may lead to death. Infants should continue to be monitored closely for enterocolitis many years after corrective surgery because the infection has been reported to occur up to 10 years later[108,109].

TREATMENT

Surgery is the treatment of choice for Hirschsprung disease. There are many common procedures. Before surgery, serial rectal irrigation helps decompress the bowel and prevent enterocolitis[108]. In healthy newborns with undistended colons and short-segment Hirschsprung's disease, the definitive procedure of ileoanal pull-through anastomosiscan be performed[110,111]. If the child has Hirschsprung's associated complication such as enterocolitis or a significantly dilated colon, a colostomy can be placed for several months while the child recovers; the pull-through procedure is performed four to six months after colostomy placement[112]. There are several pull-through techniques, with 4 to 16 percent complication. Swenson's operation involves removing the

rectum, pulling the healthy ganglionated colon through, and connecting it to the anus. Newer techniques such as Duhamel operation, Soave operation help preserve the intricate nerve supply to the rectum [113]. Dilations of the anastomosis are done for several months after the Soave operation to prevent stricture formation. This can performed by the patient's parents at home. All these procedures have high success rates, and morbidity is very minimal[114] Some surgeons do an one-stage transanal Soave operation in newborns with short-segment disease, eliminating the need for an abdominal incision and colostomy[115]

MATERIALS AND METHODS

MATERIALS AND METHODS

Material 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 from August 2016 to July 2018 in Institute of Child Health and Hospital for Children , Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai.

During our study period we received around 2,524 specimens for histopathological examination. Since it is a pediatric hospital we received 400 specimens from gastrointestinal tract and out of which we received 110 cases of clinically suspicious Hirschsprung disease. Out of 110 cases 95 cases were histopathologically diagnosed as Hirschprung disease. Out of 95 cases a conclusive opinion could not be given due to sampling error in 6 cases. Out of 95 cases, 55 cases were taken for immunohistochemistry and special stain.

INCLUSION CRITERIA

1.Cases of Hirschprung disease diagnosed by hematoxylin and eosin

2.Cases of age group less than 5 years.

EXCLUSION CRITERIA

1.Blocks with inadequate material.

2.Other causes of aganglionosis.

3. 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 were subjected for routine histopathological examination. The following clinical and pathological parameters were evaluated age, gender, complaints and site of the lesion.

Hirschsprung disease were typed according to presentation confirmed by imaging and peroperatively. Absence of ganglion cells are viewed in a meticulous way since biopsies are received from 3 sites from the same patient. The sites are transition zone, proximal colostomy site and distal colostomy site. The absence of ganglion cells confirms the diagnosis of Hirschsprung disease. While reporting presence of ganglion cells in colostomy site should be given, because the presence of ganglion cells is required for the proper functioning of colostomy stoma colostomy. Out of 110 cases, 95 cases were Hirschsprung disease, one case as intestinal neuronal dysplasia,8 cases as non Hirschsprung disease and for 6 cases opinion could not be given. In 95 cases 55 cases were analysed with immunohistochemistry and special stain.

Immunohistochemistry was done with Calretinin and S100. Special stain was done by using toluidine blue.

IMMUNOHISTOCHEMICAL EVALUATION:

Immunohistochemical analysis of calretinin & S100 were performed in paraffin embedded tissue samples by using a super sensitive polymer HRP 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 done. The antigen was bound with rabbit polyclonal antibody (Pathinsitu) against calretinin and rabbit monoclonal antibody against S 100 protein and then detected by adding secondary antibody conjugated with horse radish peroxidase-polymer and diaminobenzidine substrate.

Table 1

ANTIGEN	VENDOR	SPECIES (CLONE)	DILUTION	POSITIVE CONTROL
CALRETININ	PATHINSITU	POLYCLONAL	READY TO USE	GANGLIONEUROMA
S 100	PATHINSITU	MONOCLONAL	READY TO USE	SKIN

Procedure of IHC

1.4µ thick sections were cut from formalin fixed paraffin embedded tissue blocks and transferred into positively charged glass slides.

2. The glass slides were kept in an incubator at 58 degree Celsius overnight.

3. Deparaffinisation in xylene was done for 15 minutes and 2 changes

4. Dehydration was with absolute alcohol for 5 minutes and 2 changes

5. The sections were washed in tap water for 10 minutes.

6. Washing of sections in distilled water for 5 minutes

7. Antigen retrival was done with microwave oven with sections immersed in Tris-EDTA buffer (preheated at 800 watts for 4 minutes) for 20 minutes

A. 800 watts – 5 minutes

B. 640 watts – 10 minutes

C. 480 watts – 5 minutes

8. Slides are cooled till it comes toroom temperature and then washed with distilled water for 10 minutes

9. Washed in phosphate wash buffer for 5 minutes and 2 changes

10. Application of peroxide block was done over the sections for 5 minutes

11. Slides were washed with phosphate wash buffer for 5 minutes

12. Primary antibody was applied over the sections and incubated for 45 minutes

13. After washing in wash buffer for 5 minutes, HRP-conjugated polymer applied to the sections and incubated for 30 minutes

14. Slides were washed with 2 changes of wash buffer for 2 minutes each

15. Sections were then covered with Di-amino benzidine (DAB) chromogen (prepared by diluting 1 drop of DAB chromogen to 1ml of DAB buffer) for 5 minutes.

16. Counterstaining was done with haematoxylin, washed in running tap water, air dried, cleared with xylene and mounted.

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

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

S 100

S100 shows strong cytoplasmic positivity in submucosal nerve trunk.Focally it will be positive in normal nerve fibres,but if there are thickened nerve trunks they are hypertrophic nerve bundles which is an additional criteria for diagnosing Hirschsprung disease.thickness could be of > 40µ .

Special stain

Table 2 special stain toluidine blue

vendor	Positive control
Fischer scientific	Mast cells in GIT

Procedure of toluidine blue

Toluidine blue staining in a paraffin sections were performed using a simple method which required incubation of sections in 0.2% aqueous solution of Toluidine blue in 56ċ for 30 minutes and finally mounting in a water based medium.

Interpretation

Cytoplasm stains an ultramarine blue colour and nucleus blue colour.

This is due to the presence of RNA content which is present in the Nissl substance which is present in the cytoplasm.

STATISTICAL ANALYSIS:

The statistical evaluation was performed with 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 were analyzed and correlated with clinical variables like age, sex, clinical presentation ,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 is compared with toluidine blue and analyzed for statistical correlation. In the present study, the P value below 0.05 is considered significant.

OBSERVATION AND RESULTS

During our study period of 24 months from August 2016 to July 2018 we received around 2,524 specimens for histopathological examination in Institute of Child Health and Hospital for Children, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai.

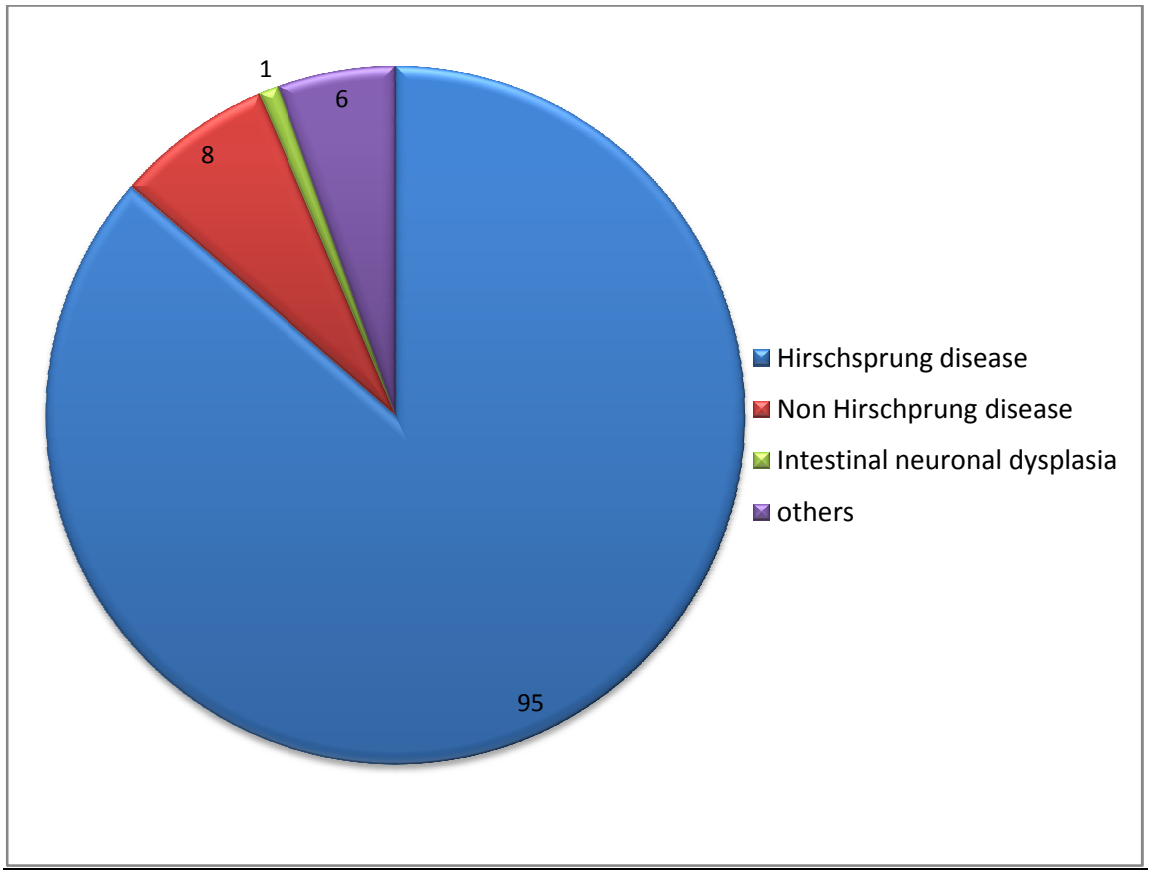
Of this, 110 cases of clinically suspicious Hirschsprung disease both colostomy and biopsy specimens were received .In this 8 suspected cases of Hirschsprung disease was found to be a non Hirschsprung disease showing the presence of ganglion cells . One case which was suspected as Hirschsprung disease 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 submucosa. A total of 55 cases which were biopsy proved were taken and analysed.

TABLE 1. SPECIMENS RECEIVED FROM CLINICALLY SUSPICIOUS HIRSCHSPRUNG DISEASE (110)

	DIAGNOSIS
HIRSCHSPRUNG DISEASE	95
NON HIRSCHSPRUNG	8
INTESTINAL NEURONAL DYSPLASIA	1
INADEQUATE MATERIAL	3
INCONCLUSIVE OPINION	3
TOTAL	110

Of 110 cases of clinically suspicious Hirschsprung disease, 8 cases showed presence of ganglion cells which excludes disease, 95 cases showed absence of ganglion cells proved to be Hirschsprung disease. One case showed the presence of ganglion cells but was not functional and hence reported as intestinal neuronal dysplasia

Chart 1- SPECIMENS RECEIVED FROM CLINICALLY SUSPICIOUS HIRSCHSPRUNG DISEASE



Among the 110 cases of clinically suspected Hirschsprung disease 95 were confirmed by histopathological examination, 3 were inconclusive as the specimen had only mucosa . For a proper histopathological examination sub mucosa should be examined. Material were inadequate in 3 cases.

OBSERVATION AND RESULTS

AGE WISE DISTRIBUTION

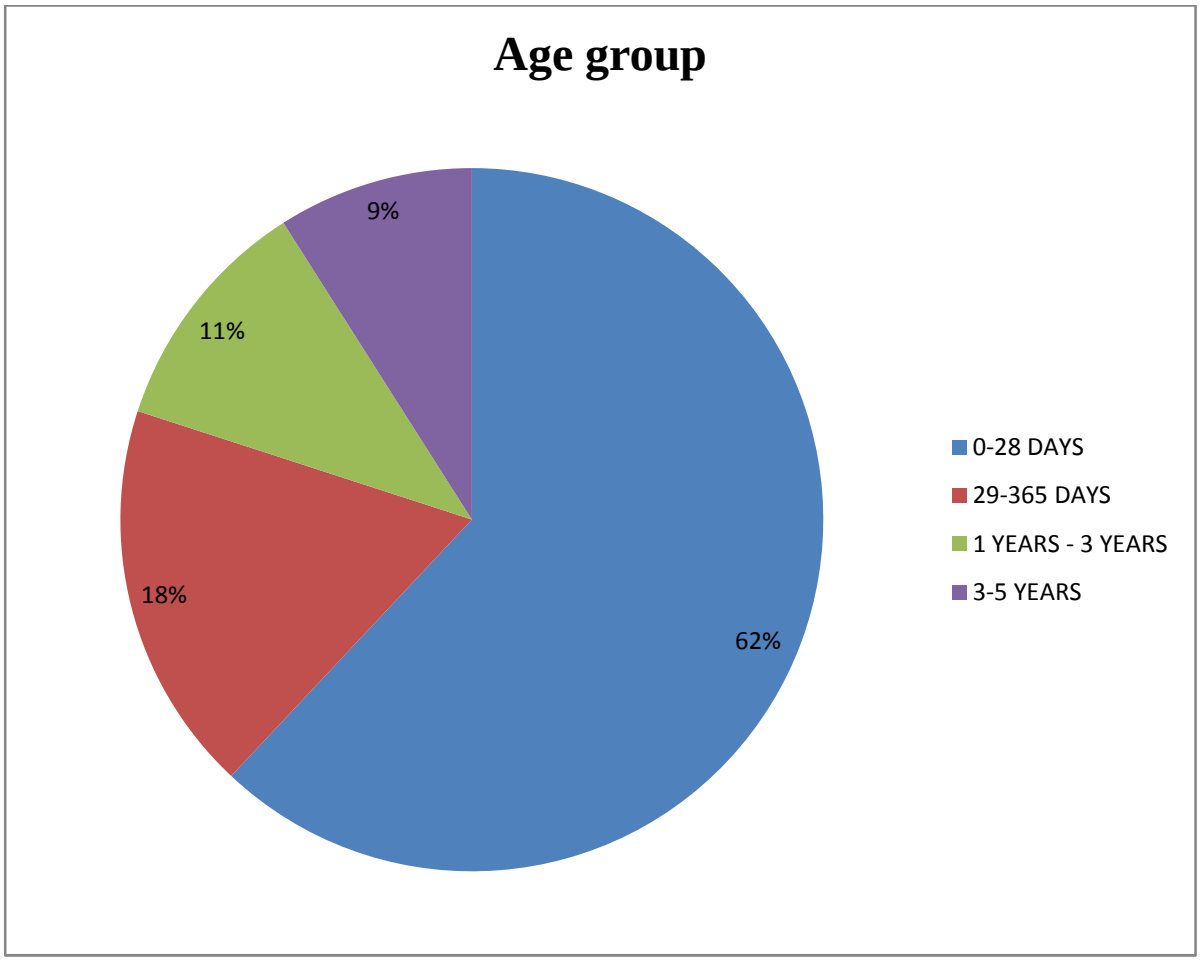
TABLE 2.SHOWING AGE WISE DISTRIBUTION OF CASES

(55 CASES STUDY)

AGE GROUP	Frequency	Percentage
0-28 DAYS	34	62%
29 days -1 YEAR	10	18%
1 YEARS - 3 YEARS	6	11%
3-5 YEARS	5	9%
Total	**55**	**100.0%**

Among 55 cases taken up for analysis ,34 cases are from 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 under one year of age group. Cases from later age group has reduced incidence

CHART 2: SHOWING AGE INCIDENCE



Age incidence of 55 cases of study 62% were within first 28 days of the life which is the neonatal period,

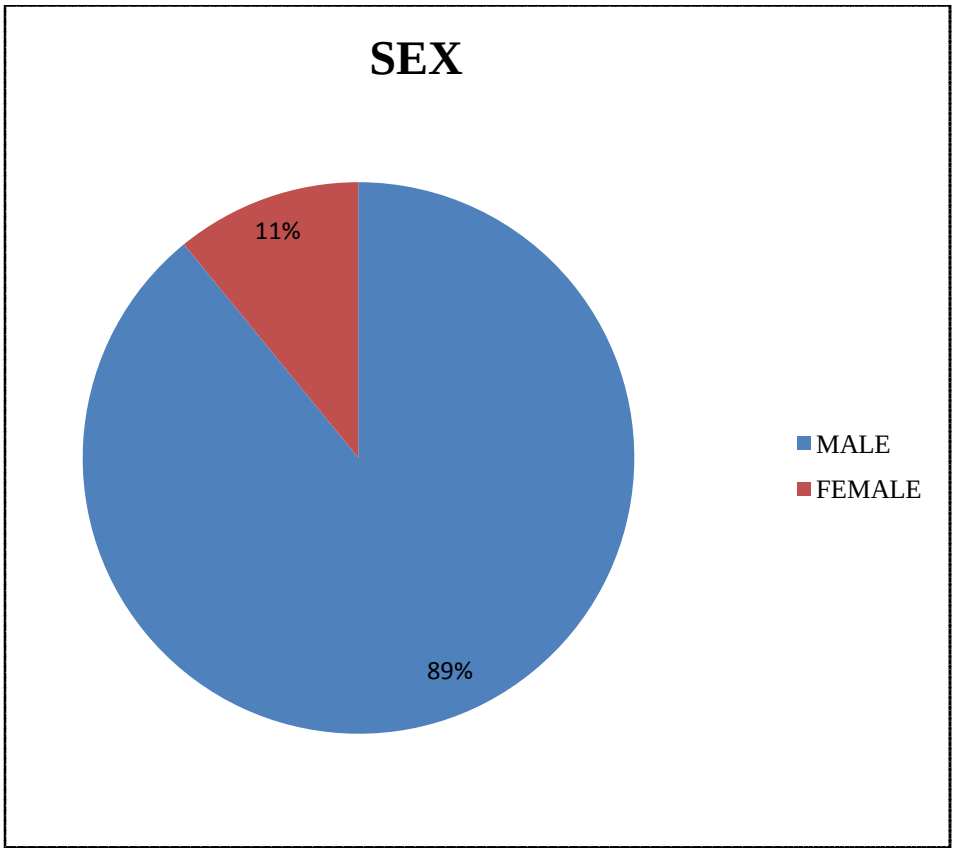
SEX WISE DISTRIBUTION

TABLE 3 : SHOWING SEX DISTRIBUTION

SEX	Frequency	Percentage
MALE	49	89%
FEMALE	6	11%
Total	**55**	**100%**

Among 55 cases of Hirschsprung disease, 49 cases were male children and remaining 6 were female cases. This finding correlates with literature where male patients predominate in disease manifestation.

CHART 3: SHOWING SEX DISTRIBUTION



Among the 55 cases, 89% cases were male children and remaining 11% were female children

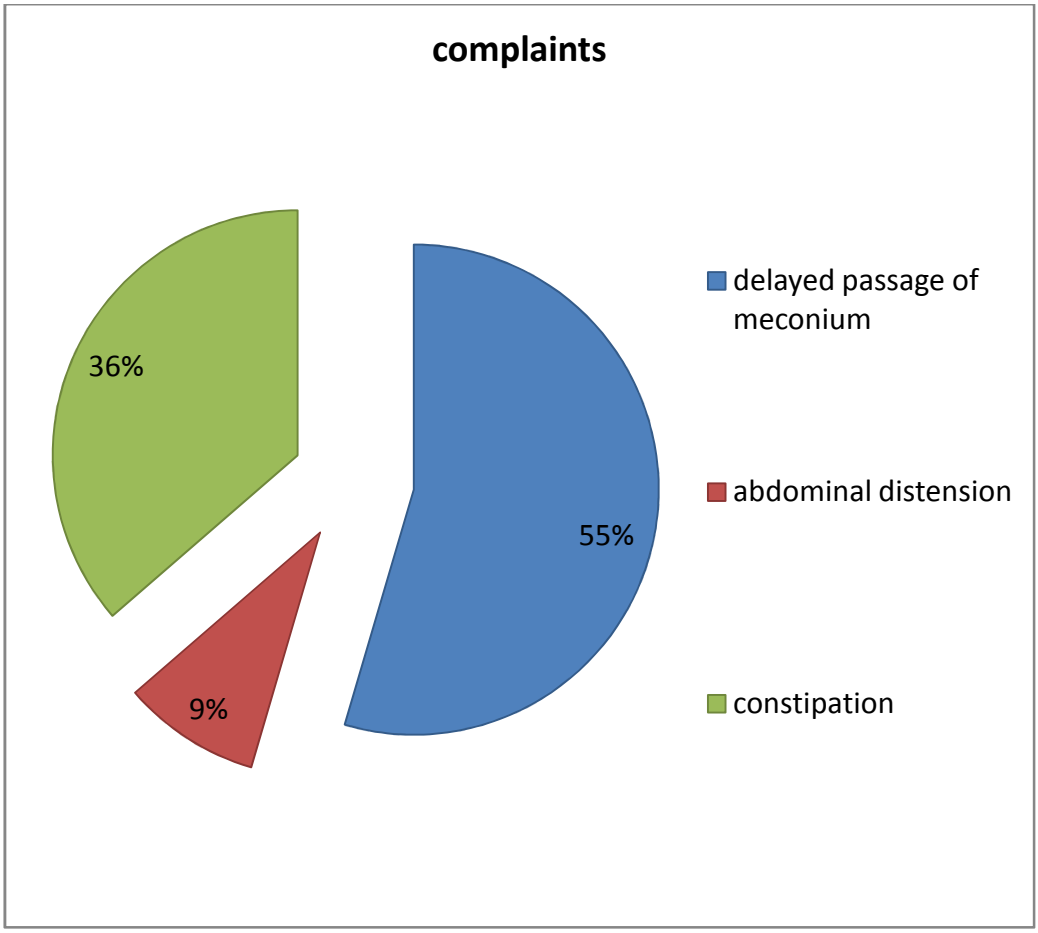
CLINICAL PRESENTATION

TABLE 4. SHOWING THE CLINICAL PRESENTATION

COMPLAINTS	FREQUENCY	PERCENTAGE
DELAYED PASSAGE OF MECONIUM	30	55%
ABDOMINAL DISTENSION	5	9%
CONSTIPATION	20	36%

Among 55 cases majority of cases were new born presenting with the complaints of delayed passage of meconium. Next mode of presentation in the new born was abdominal distension. Constipation is the presenting complaint in children of age more than one year.

CHART 4: SHOWING THE DISTRIBUTION OF CLINICAL PRESENTATION



Delayed passage of meconium and abdominal distension is found to be the most common presentation of Hirschsprungdisease,since it is a disease of which is presenting 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.

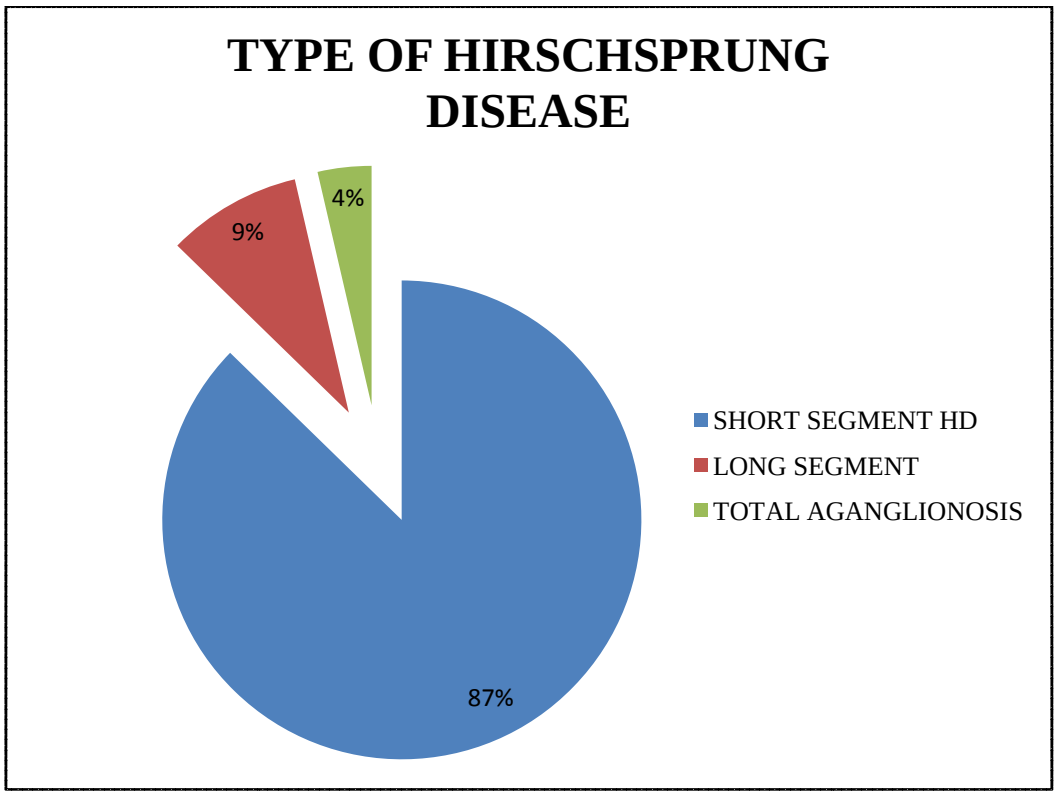
TYPES OF HIRSCHSPRUNG DISEASE

TABLE 5: TABLE SHOWING TYPES OFHIRSCHSPRUNG DISEASE

TYPE OF DISEASE	FREQUENCY	PERCENTAGE
SHORT SEGMENT HD	48	87%
LONG SEGMENT	5	9%
TOTAL AGANGLIONOSIS	2	4%

Among the 55 cases we had around 48 cases presenting as with short segment disease with rectosigmoid involvement of disease, next most common presentation is the long segment disease. Finally we had two cases of total aganglionosis which is associated with very grave prognosis.

CHART 5 : CHART SHOWING TYPE OF HIRSCHSPRUNG DISEASE



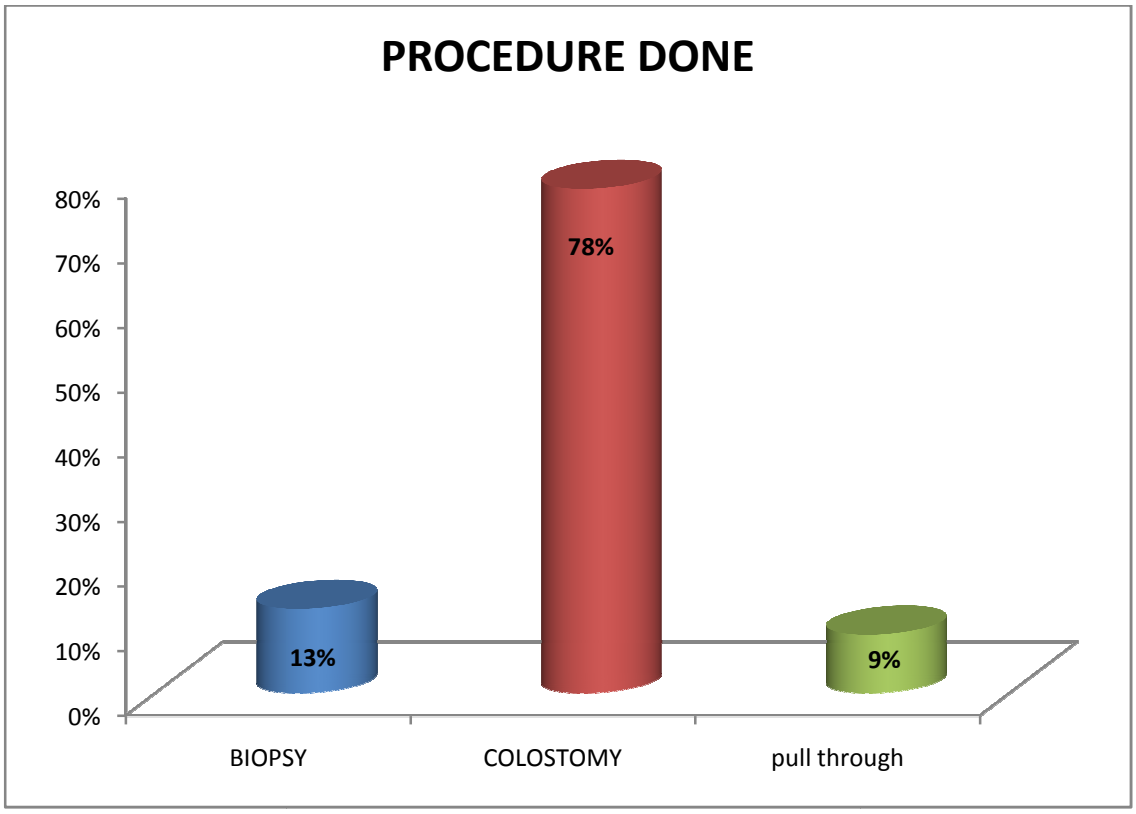
The most common type encountered in our study was short segment disease with rectosigmoid presentation followed by long segment disease. Two cases of total aganglionosis were seen with involvement of total bowel and associated with grave prognosis.

TABLE 6: TABLE SHOWING THE TYPES OF PROCEDURE DONE FOR HIRSCSPRUNG DISEASE

PROCEDURE DONE	Frequency	Percentage
BIOPSY	7	13%
COLOSTOMY	43	78%
Duhamel- pull through	5	9%
Total	55	100%

Among the 55 cases ,we had 43 cases received by means of colostomy procedure and 5 cases were with Duhamel pull through procedure and 7 cases seromuscular biopsy. Seromuscular biopsy is done when there is a strong clinical suspicion of disease. Duhamel pull through is done after the colostomy procedure in a period of gap.

Chart 6: CHART SHOWING THE TYPES OF PROCEDURE DONE FOR DISEASE



Among 55 cases 78% of cases were from colostomy procedure and 9% from Duhamel pull through and around 13% were seromuscular biopsy.

IMAGING FINDINGS

All the cases basically present with delayed passage of meconium and furthur evaluation is done by means of imaging. Initial imaging could be done both by ultrasonogram and x ray, further confirmed by barium contrast study.In ultrasonogram and x ray the common finding is the dilated bowel loops.In our hospital nearly all cases were diagnosed by x ray finding with dilated bowel loops.

TABLE 7: TABULAR COLUMN OF IMAGING FINDINGS

	Frequency	Percent	Valid Percent	Cumulative Percent
DILATED BOWEL LOOPS	55	100.0	100.0	100.0

HISTOPATHOLOGICAL EXAMINATION

Initial diagnosis was made using H& E stained paraffin sections by absence of ganglion cells. immunohistochemistry was initially not done.

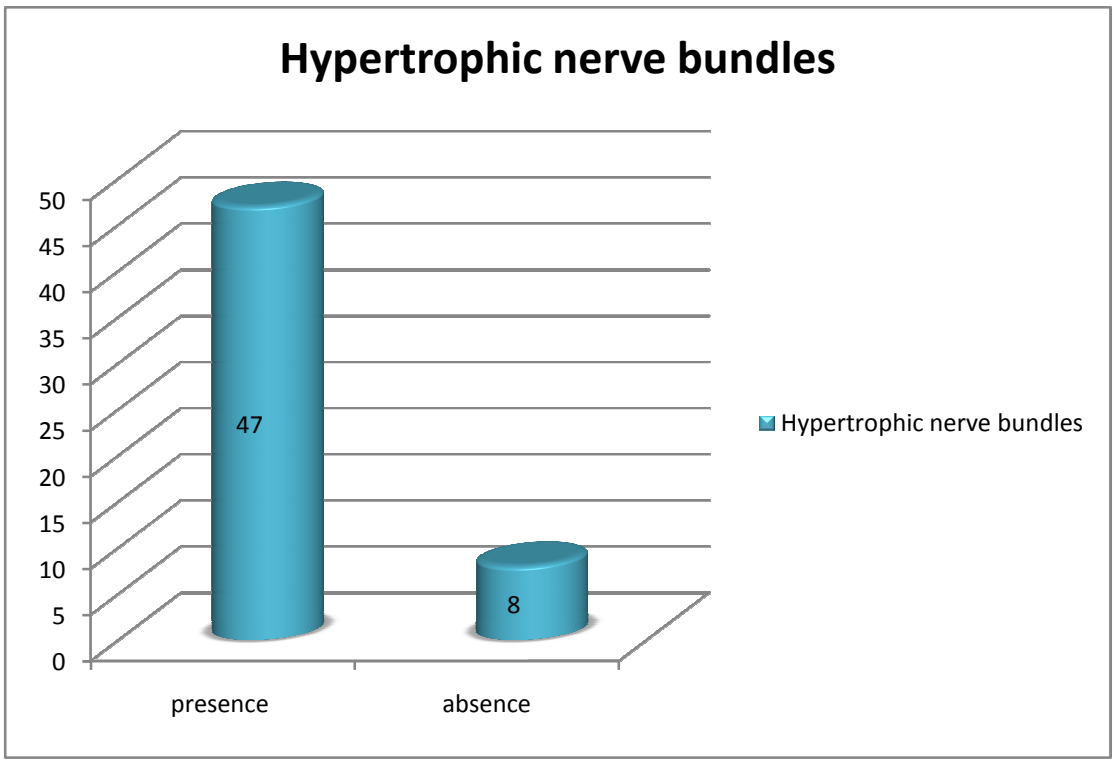
TABLE : 8 TABLE SHOWING ABSENCE OF GANGLION CELLS

	Frequency	Percent	Valid Percent	Cumulative Percent
ABSENCE OF GANGLION CELL	55	100.0	100.0	100.0

Hypertrophic nerve bundles in histopathological examination

Presence of hypertrophic nerve bundles is an additional finding in Hirschsprung disease. But its absence does not exclude the diagnosis. When it is present along with the absence of ganglion cells it confirms the diagnosis of disease. In our study we had hypertrophic nerve bundles seen in 47 cases and only 8 ccases showed absence of hypertrophic nerve bundles.

CHART 7 HYPERTROPHIC NERVE BUNDLES



RESULTS OF IMMUNOHISTOCHEMICAL STUDIES

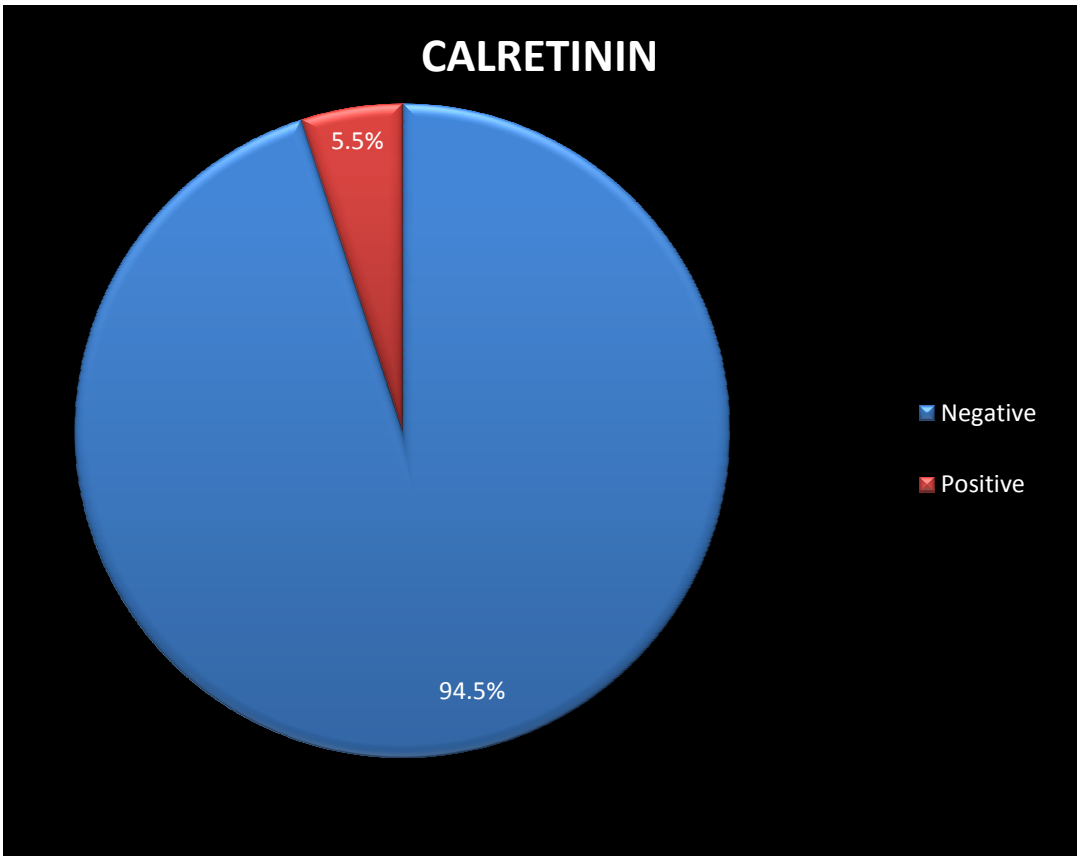
IMMUNOHISTOCHEMICAL EXPRESSION OF CALRETININ

Table 9. Expression of calretinin

CALRETININ	Frequency	Percentage
Negative	52	94.5%
Positive	3	5.5%
Total	55	100.0%

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 missed in HPE. Immunohistochemistry confirms the presence of ganglion cells since calretinin was positive.

CHART : 8 EXPRESSION OF CALRETININ



Among the 55 cases of histopathologically diagnosed Hirschsprung disease 3 cases were showing calretinin positive which is about 5.5% in total. Calretinin is a marker of ganglion cells. It confirms the presence of ganglion cell which is against the histopathological diagnosis.

IMMUNOHISTOCHEMICAL EXPRESSION OF S 100

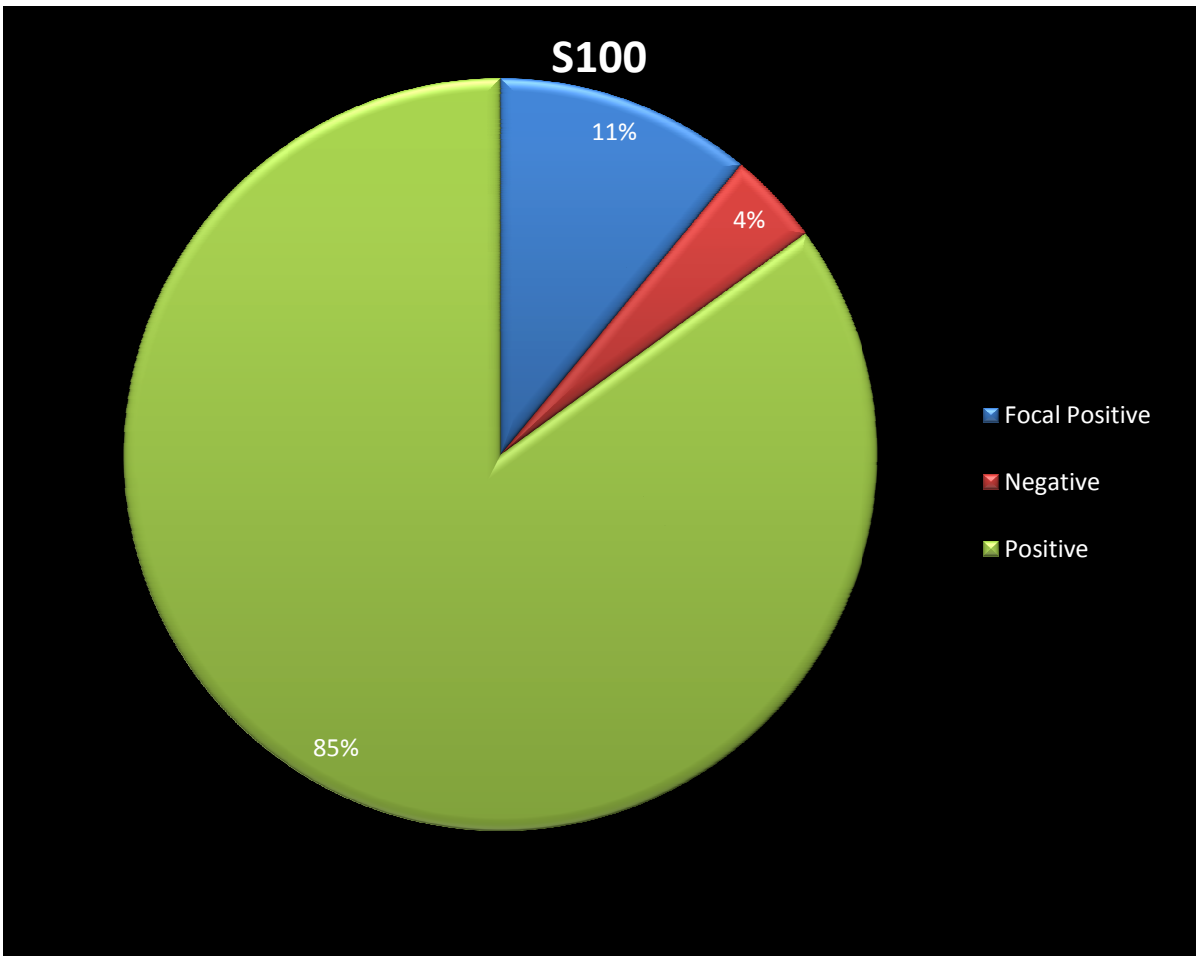
S 100 indicates the presence nerve fibres, positivity denotes there are thickened nerve bundles, focal positive denotes there are no thickened nerve bundles ,just nerve fibres are present.

TABLE 10 : IMMUNOHISTOCHEMICAL EXPRESSION OF S 100

S 100	Frequency	Percentage
focal positive	6	11%
negative	2	4%
positive	47	85%
Total	55	100%

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 S 100 was negative.

CHART : 9 IMMUNOHISTOCHEMICAL EXPRESSION OF S100



OUT OF 55 CASES 85% showed positive and 11% showed focal positivity and 4% is negative. It is not a confirmatory test and it is just an additional finding.

SPECIAL STAIN TOLUIDINE BLUE EXPRESSION

Toluidine blue special stain was used here for the identification of ganglion cells.

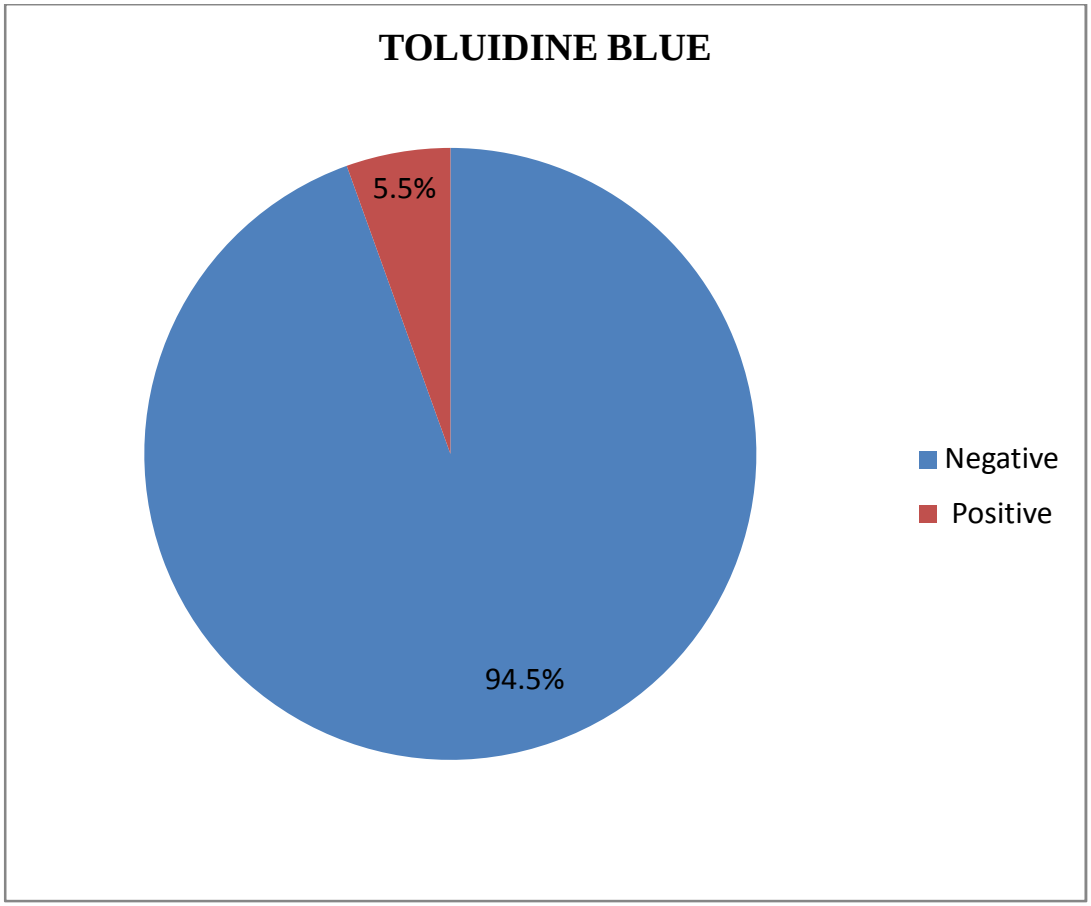
In 55 cases, 3 cases showed positivity which also helps in diagnosing ganglion cells.

Toluidine blue stain can also be used for visualizing the hypertrophic thickened nerve fibres. In sections stained with toluidine blue mast cells are also seen.

TABLE 11.TOLUIDINE BLUE EXPRESSION

TOLUIDINE BLUE	FREQUENCY	PERCENTAGE
NEGATIVE	52	94.5%
POSITIVE	3	5.5%
TOTAL	55	100%

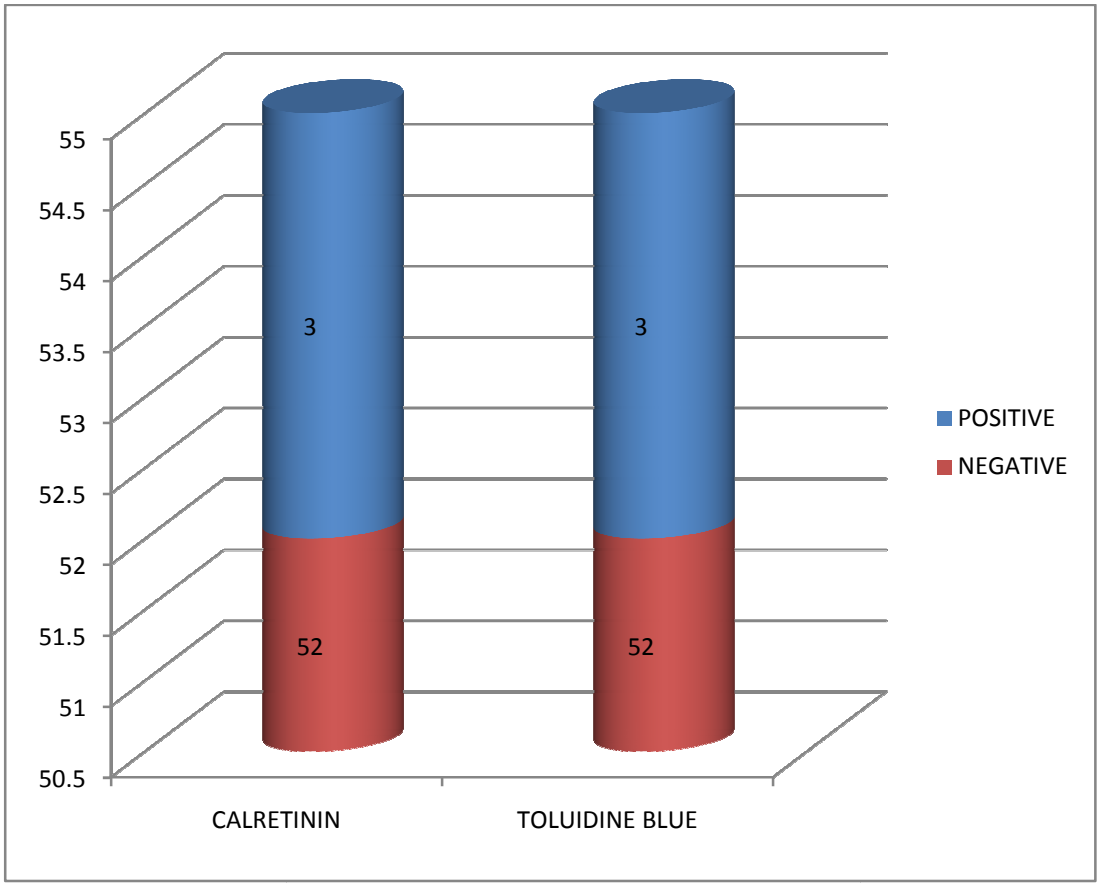
Chart 10 : TOLUIDINE BLUE EXPRESSION



Toluidine Blue special stain showed 5.5% positivity.

COMPARISION OF CALRETININ WITH TOLUIDINE BLUE

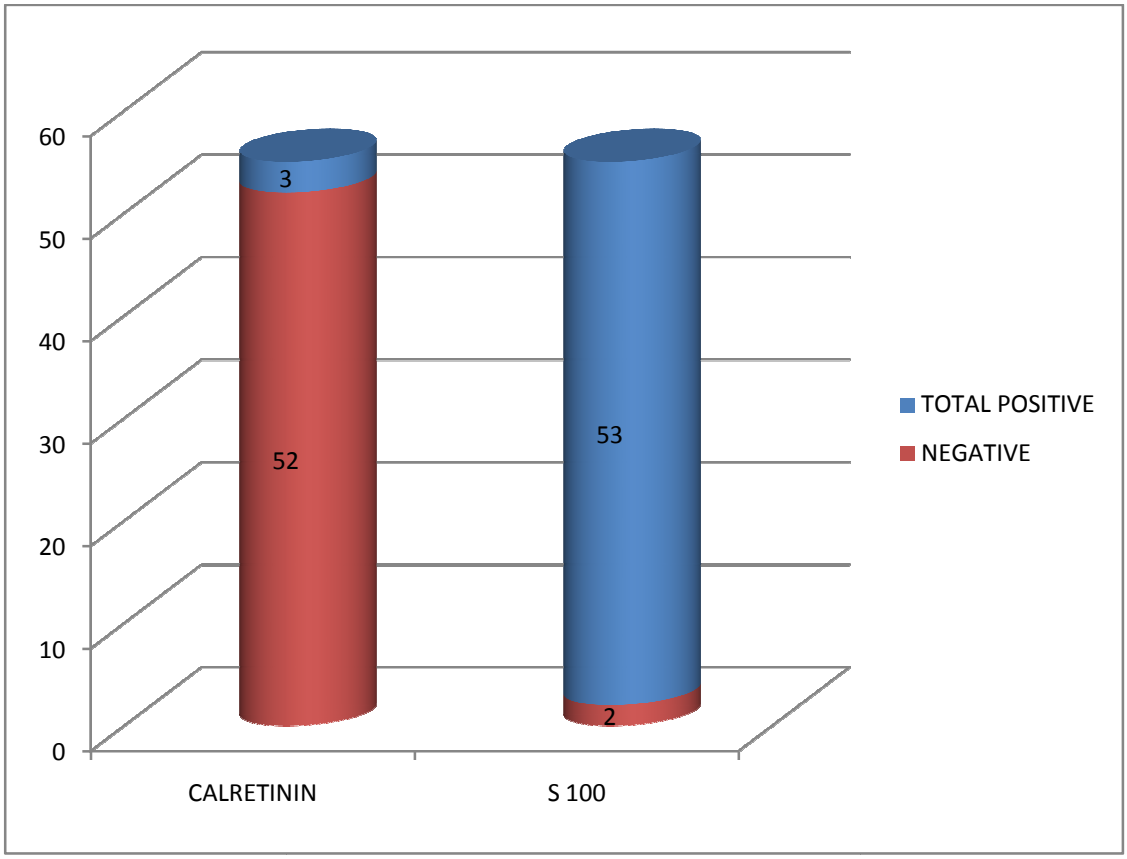
CHART 11 : SHOWING COMPARISION OF TOLUIDINE BLUE WITH CALRETININ



Calretinin positive in 3 casesand Toluidine blue positive in 3 cases . Both stains aid in identifying the ganglion cells.

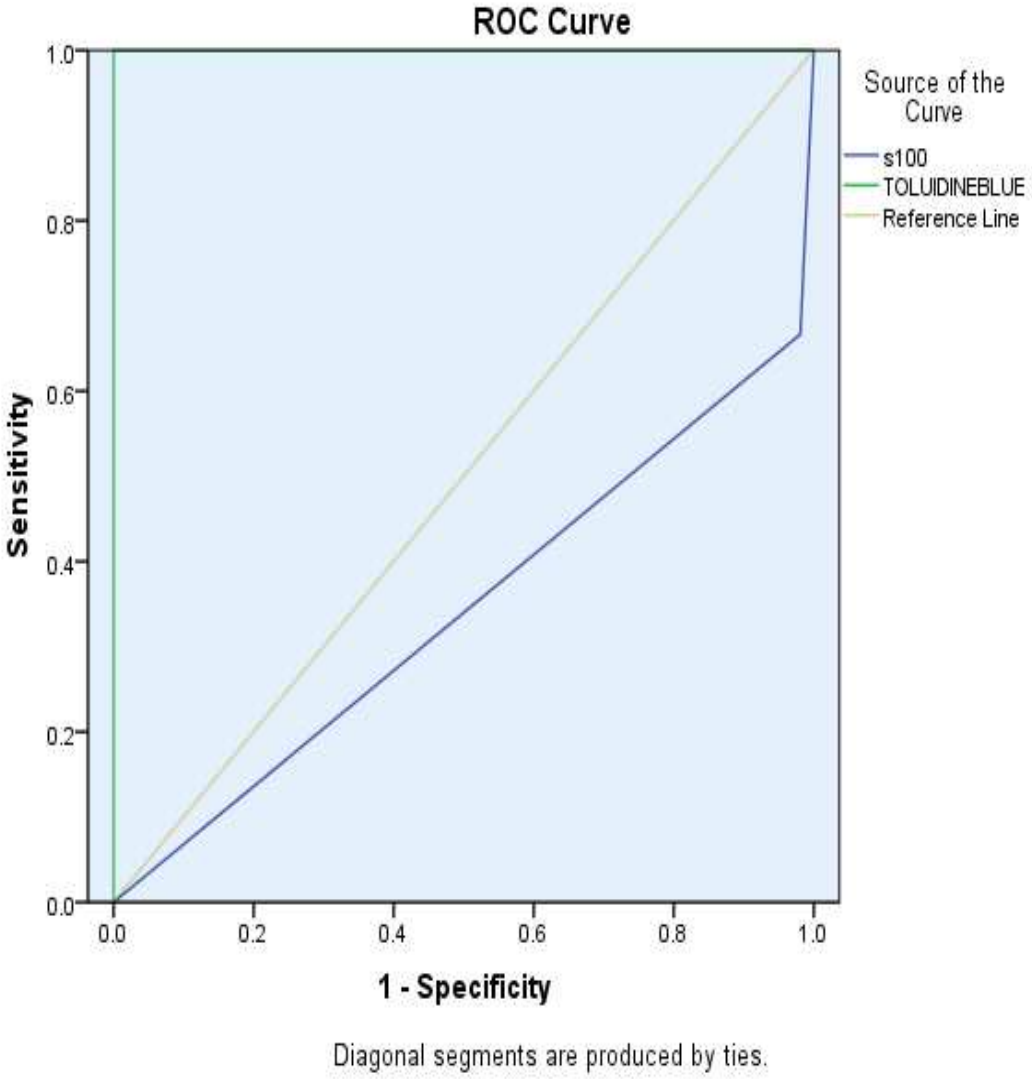
COMPARISON OF CALRETININ AND S100

CHART 12 : SHOWING COMPARISION OF TOLUIDINE BLUE WITH CALRETININ



Calretinin negative in 52 cases and S100 positive out of 53 cases.

ROC CURVE


ROC Curve
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Source of the Curve
s100
TOLUIDINEBLUE
Reference Line
Diagonal segments are produced by ties.

Area Under the Curve

Test Result Variable(s)		Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
					Lower Bound	Upper Bound
dimension 0	s100	.343	.194	.364	.000	.784
	Toluidine blue	1.000	.000	.004	1.000	1.000

The test result variable(s): S100 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Crosstab

			CALRETININ		Total
			POSITIVE	NEGATIVE	
S100	POSITIVE	Count	2	51	53
		% within CALRETININ	66.7%	98.1%	96.4%
	NEGATIVE	Count	1	1	2
		% within CALRETININ	33.3%	1.9%	3.6%
	Total	Count	3	52	55
		% within CALRETININ	100.0%	100.0%	100.0%

Pearson Chi-Square=7.986** P<0.001

Crosstab

			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%

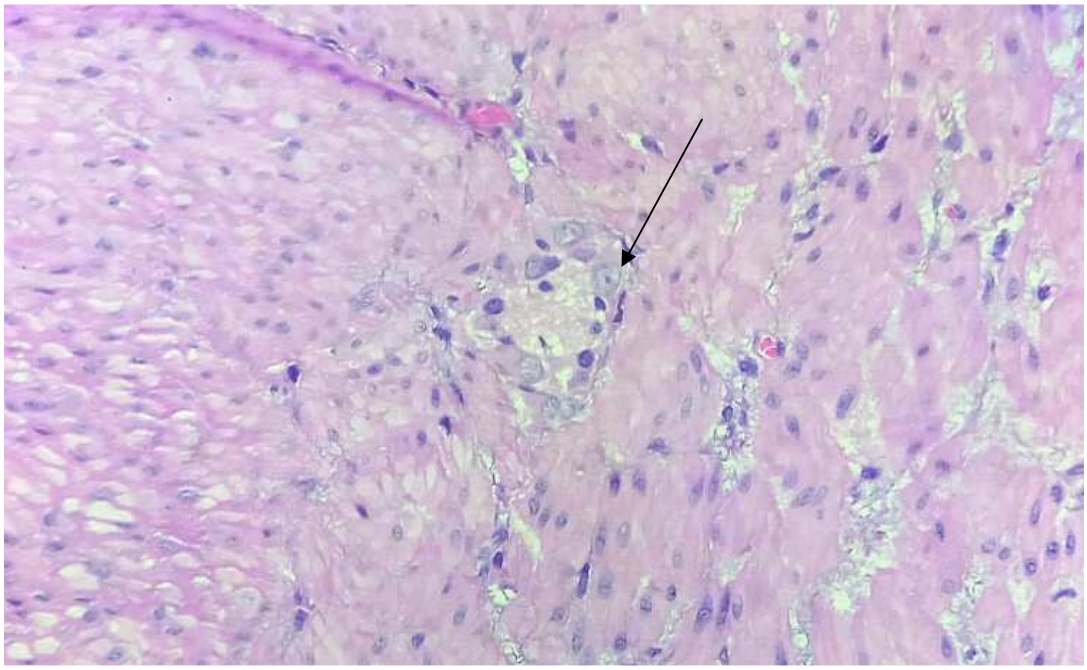
Pearson Chi-Square=55.00** P<0.001

p value of s100 – 0.364

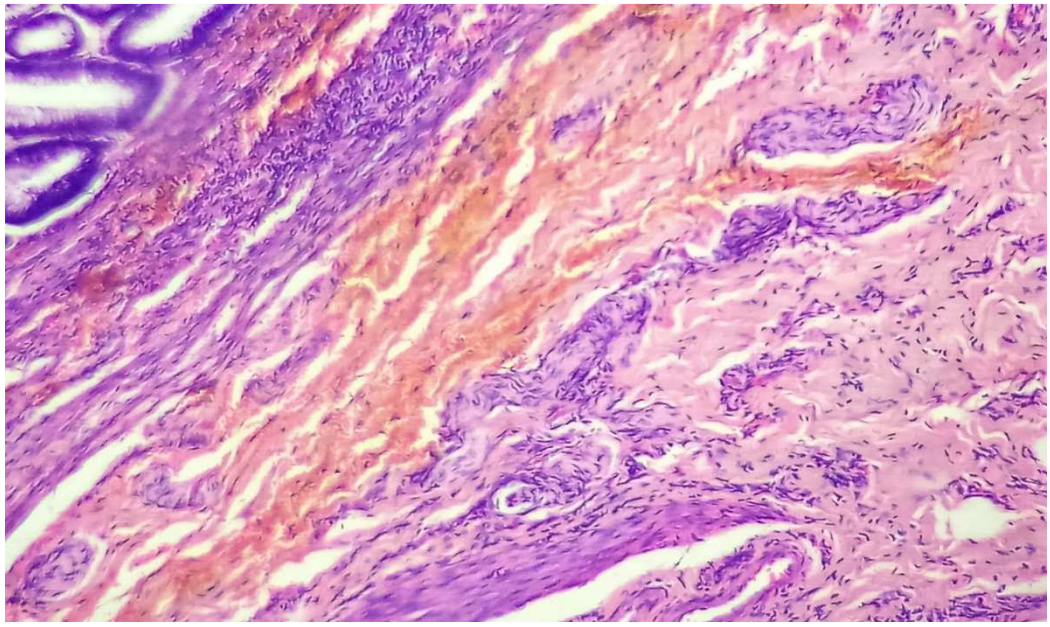
p value of toluidine blue -0.004

The results with calretinin and toluidine blue are comparable and the results are statistically significant (< 0.001) 100% sensitive but specificity cannot be arrived since it needs larger samples. If number of cases increases with larger samples then we can conclude that toluidine blue is a better method. But in our conclusion toluidine blue is equally good as calretinin.It can be substituted in place of calretinin where if it is not available.

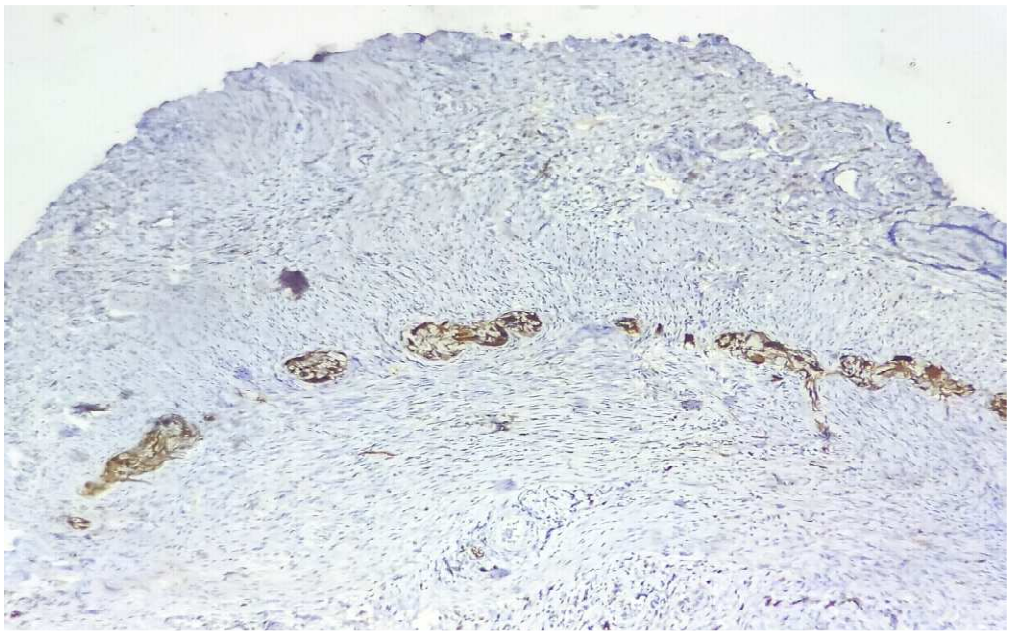
COLOUR PLATES



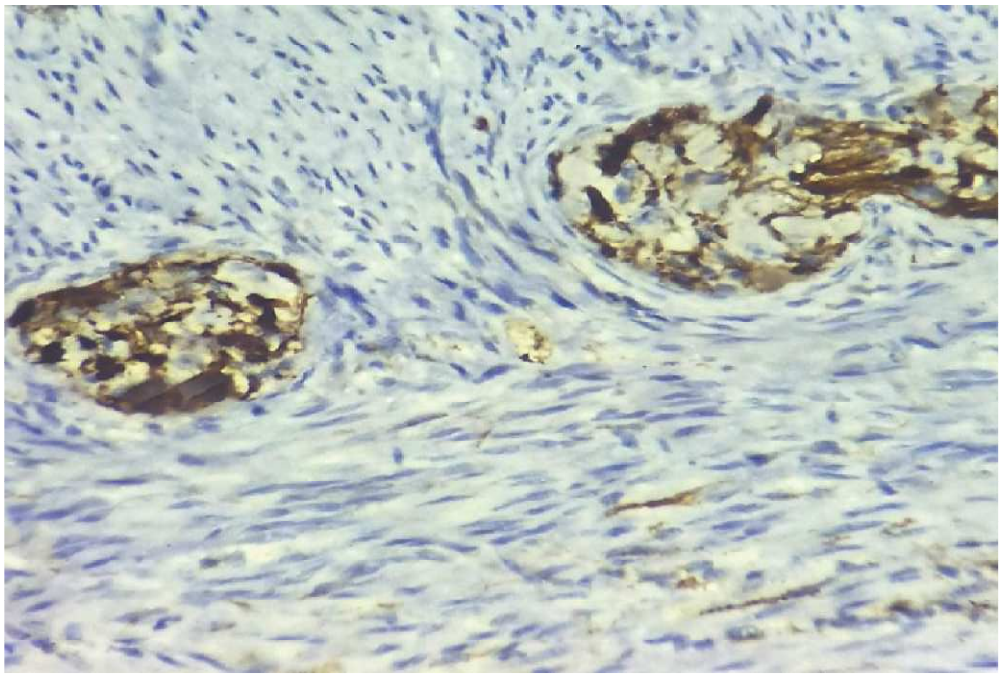
400 X (H & E) Showing Presence of Ganglion Cells



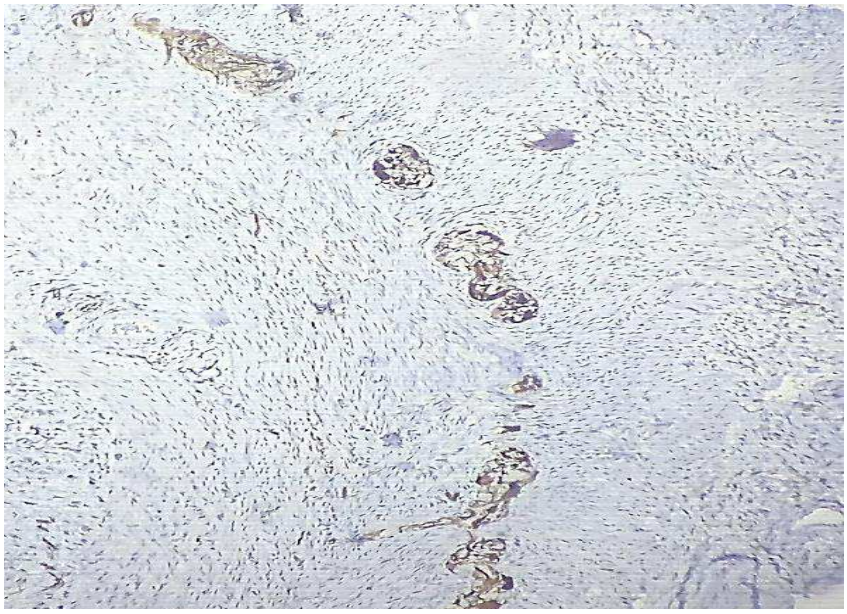
100 X (H & E) Showing nerve bundle hypertrophy



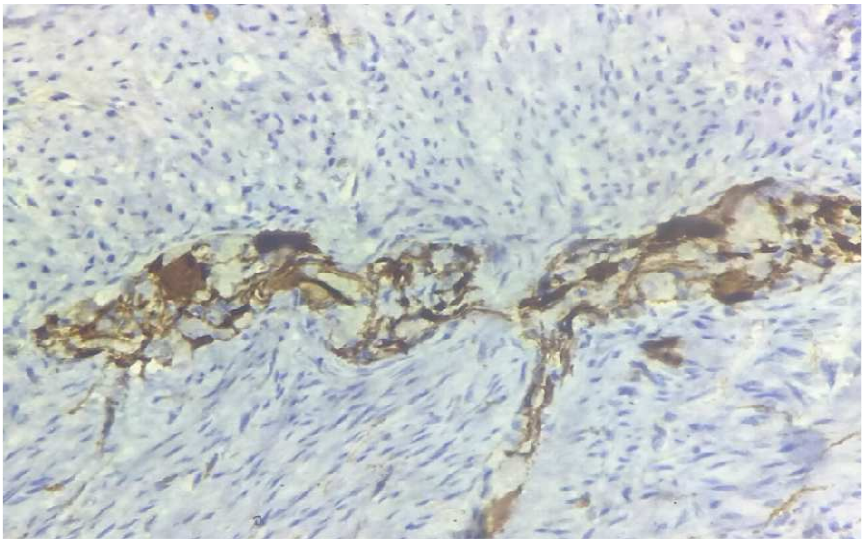
100 X (IHC) Showing Calretinin Positive



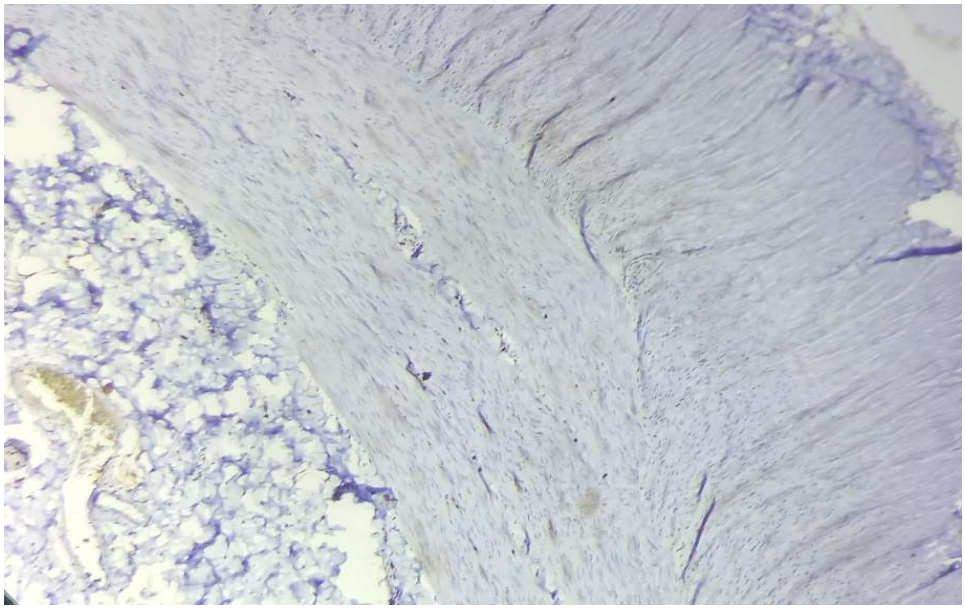
400 X (IHC) Showing Calretinin Positive



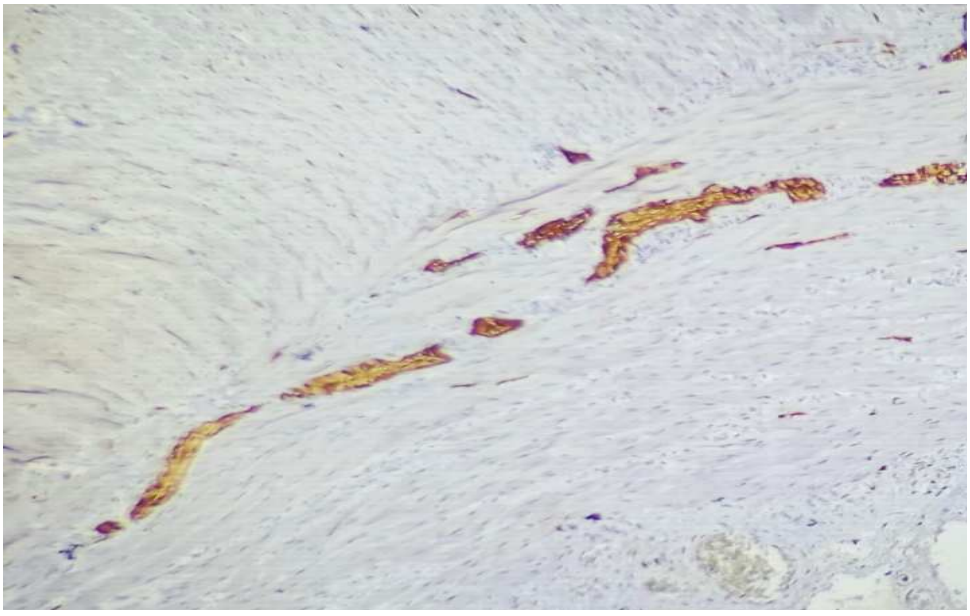
100 X (IHC) Showing Calretinin Positive



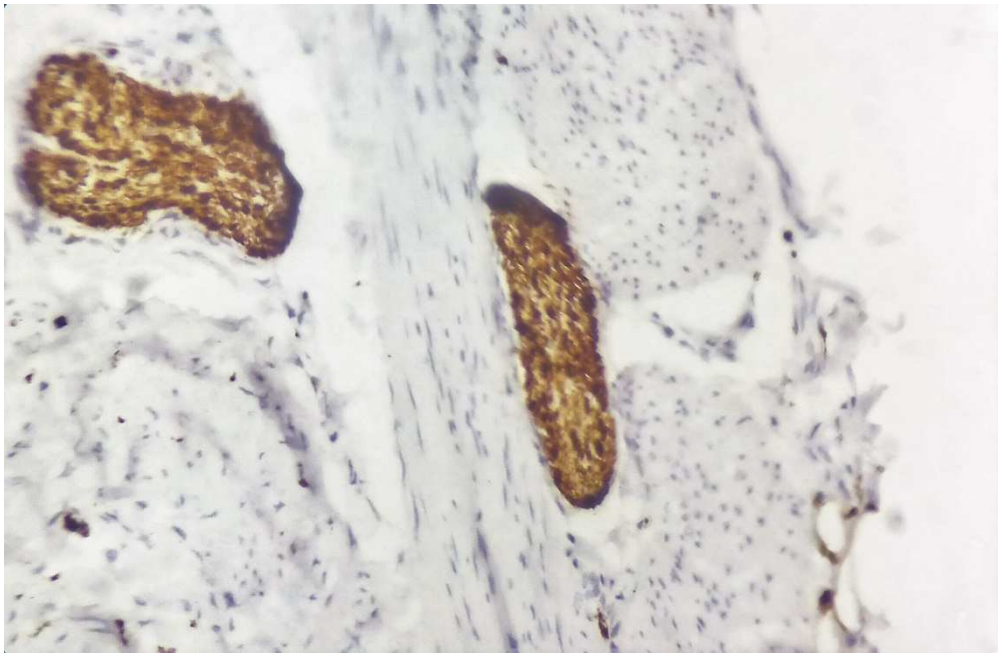
400 X (IHC) Showing Calretinin Positive



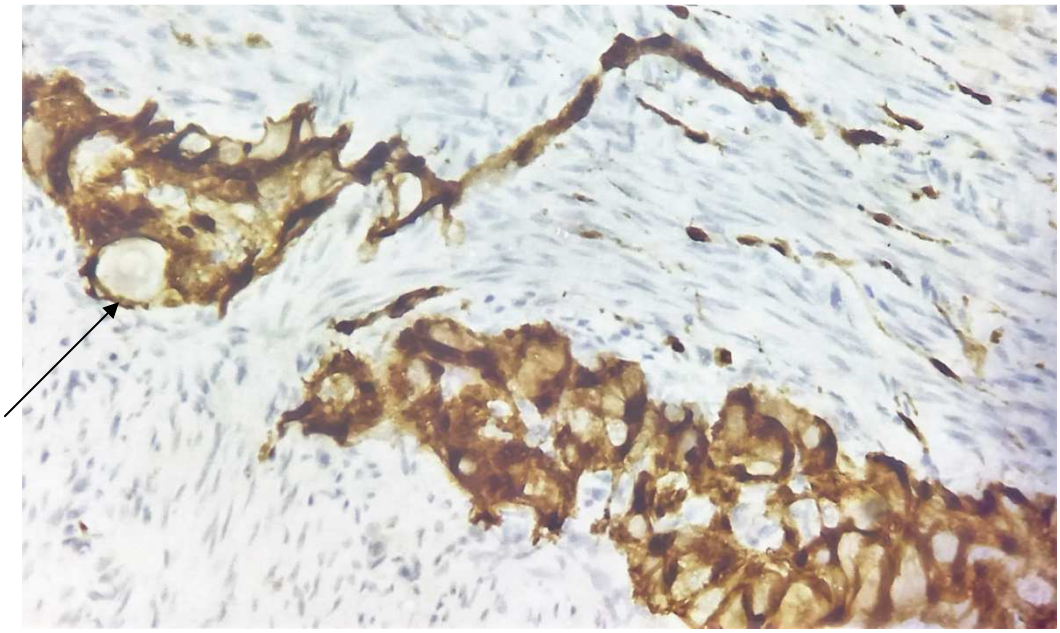
100 X (IHC) Showing Calretinin Negative



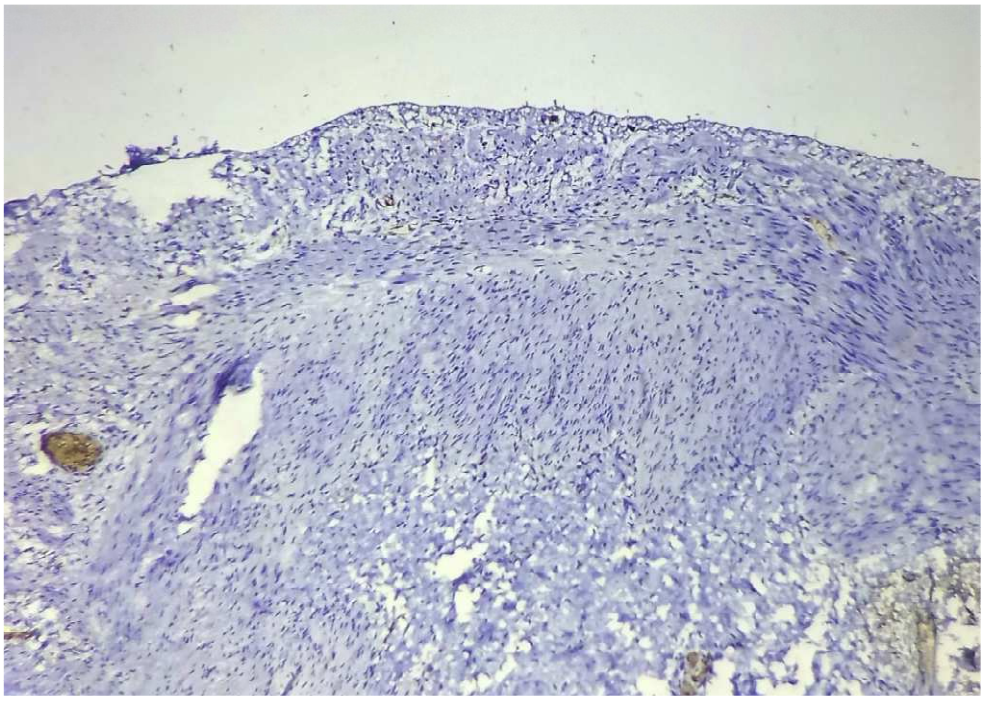
100 X (IHC) Showing Focal S 100 Positive



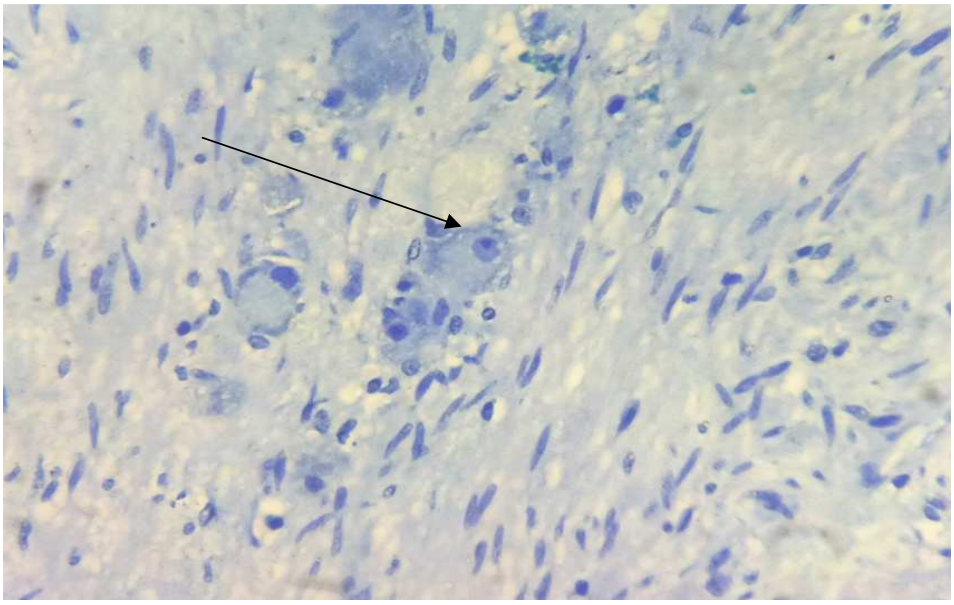
400 X (IHC) Showing Thickened Nerve Bundles in S100



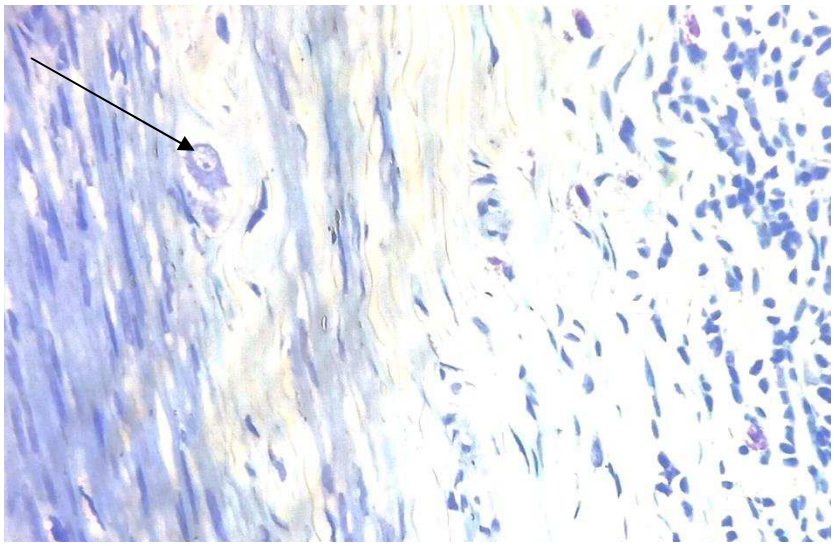
400X (IHC) Showing S 100 Negative Staining of Ganglion Cells



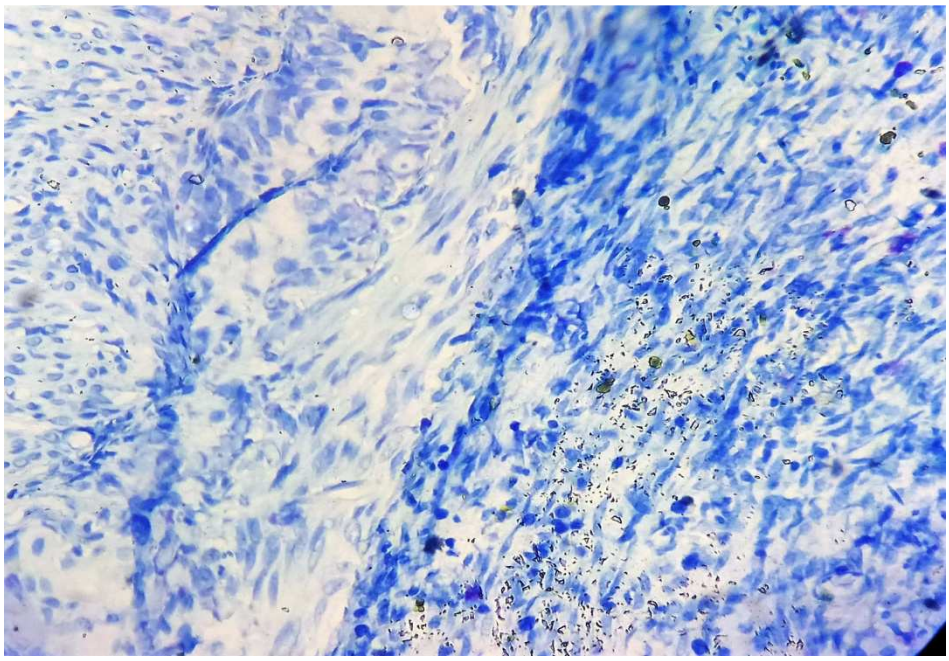
100X (IHC) Showing Focal S 100 Positive



400X Toluidine Blue Showing Positive in Ganglion Cells



400X Toluidine Blue Stain Showing Positive in Ganglion Cells



400X Toluidine Blue Stain Showing Positive in Ganglion Cells

DISCUSSION

DISCUSSION

Hirschsprung disease is common in new born and it is more common in the male children presenting more frequently in less than 1 year of age group[116,117,118,119]. As similar to many other studies our study also had 49 out of 55 cases presenting in less than one year age group.

Spouge D et al showed that new born male were most commonly affected by disease[116] Goldberg EL et al showed similar results[117].

Ikeda K et al studied in 1628 cases of disease and concluded that males are more commonly affected than females[118].

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.

Holschneider et al described short segment disease as the most common presentation[68].In our study also 87.3% were of short segment type.

In our study as our institution is a pediatric hospital we had a variety of presentation of Hirschsprung disease. Out of 110 clinically suspected cases of Hirschsprung disease only 95 were histopathologically proved Hirschsprung disease. we included cases taken cases of disease in age group less than 5 years and we analysed about 55 cases. 55 cases have been analysed both by using Immunohistochemistry and special stain. In immunohistochemistry calretinin and S 100 were used. Calretinin to identify ganglion cells and S 100

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

Calretinin 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 Barshack et all , 54 paraffin blocks were taken -24 from ganglionic segment, 17 from aganglionic segment and 13 from transtitional zone .10 out of thirteen cases showed ganglion cells with calretinin positivity. which is around 80%[89]

Zuikova et all performed a study on40 cases of Hischsprung disease and it showed a sensitivity of 100 % to calretinin [91].

Małdyk et al. reported a study in 2014 in about 14 cases of suspected Hirschsprung disease 11 cases showed absence of ganglion cell positivity and confirmed by calretinin immunostaining and it showed 99.1% sensitive[92]

Guinard Samuel et al studied in nearly 130 cases and showed nearly 100% sensitive[9.]

Hiradfar et al studied in 50 cases and showed a sensitivity of 93.3%[95]

Mukhopadhyay et al. reported that sensitivity of calretinin immunohistochemistry for ganglion cells detection was 100% and they studied in 105 cases[96]

Kaçar et al., showed calretinin was found to be highly sensitive and specific in diagnosing Hirschsprung disease in their study in 43 cases[10].

Gonzalo et al. 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 cases study with 40 cases of Hirschsprung disease[97].

Kannaiyan, *et al.*: Calretinin was extremely useful in solving the suspicious and indeterminate cases of HD. It can serve as a valuable cost-effective diagnostic aid in the centers where acetylcholinesterase enzyme histochemistry is not available[90].

Anbardar MH et al.calretinin IHC is a very consistent ,useful marker for diagnosing Hirschprung disease[120]

Kapur et al showed in his studies that calretinin appears to be a reasonable marker for evaluating Hirschprung disease[88].

Luis et al studied 14 cases with each having 50 section and he said that calretinin demonstrated a great sensitivity, specificity in identifyin g the ganglion cells.

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 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..

False positive interpretation of HD in biopsy can be given due to following factors

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

4)observer error due to lack of experience

In such occasion calretinin can be used for confirming the diagnosis of Hirschsprung disease

Table 1 calretinin in Hirschsprung disease

STUDY	SAMPLE SIZE	IHC POSITIVE PERCENTAGE
BARSHACK et al ,	54	80%
MAŁDYK et al	14	99.1%
ZUIKOVA et al	40	100%
GUINARD SAMUEL et al	130	100%
HIRADFAR et al	50	93.3%
MUKHOPADHYAY et al.	105	100%
KAÇAR et al.,	43	100%
GONZALO et al	48	100%
KANNAIYAN et al	36	100%
ANBARDAR MH et al	55	100%
KAPUR et al	31	100%
LUIS et al	14	100%

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 giving an 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 S 100

S 100 is not used as a diagnostic tool for Hirschsprung disease but is can be used as an additional criteria for diagnosing disease. Since S 100 stains the nerve fibres ,it was used to identify the thickened nerve bundles . S 100 also highlights the ganglion cells by negative staining surrounded by Schwann cells and glial fibres.

In Holland et al studies -138 cases were studied and 81% showed thickened nerve fibres[12]

In Luis et al studied 14 cases with each having 50 section showed low sensitivity of 41.7%[13]

In a study by Barshack et all , 54 paraffin blocks were taken -24 from ganglionic segment, 17 from aganglionic segment and 13 from transtitional zone ,10 out of thirteen cases showed ganglion cells presence with calretinin positivity.which is around 80% and also S 100 showing thickened nerve bundles with 100% sensitivity[89].

Table 2 : immunohistochemical evaluation of S 100

STUDY	SAMPLE SIZE	SENSITIVITY
HOLLAND et al	138	81%
LUIS et al	14	41.7%
BARSHACK et al	54	100%
IN OUR STUDY	55	85.5%

TOLUIDINE BLUE SPECIAL STAIN

Toluidine blue special stain is the one used for identifying mast cells by the property of metachromasia, but in one study in a pediatric hospital toluidine blue has been used as 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.

HA Yasseen et al studied in a total of 50 non selected cases biopsied for suspected HD which were stained with H&E stain. Based on the findings of H&E stained sections of rectal biopsies, they were divided into two groups: 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% cases[15].

Canil et al their study stated that toluidine blue method is a reproducible and reliable way of demonstrating ganglion cells in frozen rectal biopsies[106].

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 gold standard, results are compared with that of results of toluidine blue. Toluidine blue is positive in three cases where the IHC calretinin were also positive. On comparision both showed similar results . As per statistical analysis also it is statistically significant showing p value < 0.001.

Inspite of similar results toluidine blue cannot be said as superior to IHC.

Advantages of toluidine blue are

1. It is cost effective
2. The results can be obtained faster.
3. It doesnot require a well equipped laboratory set up Disadvantage of this test is that there are only limited studies for this test -one is in a paraffin embedded section and another study is the frozen rectal biopsy. Still many studies with huge sample size should be done.

Sensitivity is good but to know the specificity we need studies in a large sample.

The conclusion of the study is that toluidine blue can be used as an alternate for IHC in centres where immunohistochemistry is not available.

SUMMARY

SUMMARY

- For the study period of two years from August 2016 to July 2018, a total of 2564 specimens were received in th e Institute of Child Health and Hospital for Children , Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai for histopathological examination.
- 95 cases of Hirschsprung disease has been confirmed by histopathological examination out of 110 clinically suspicious cases.
- 34 out of 55 cases were present infirst 28 days of life (ie –neonatal period)
- 44 cases out of 55 were present in first one 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% cases presented as a short segment disease
- 2 out 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 sub mucosa.
- On immunohistochemical evaluation with calretinin 3 out of 55 showed positivity which implies that there is presence of ganglion cells.
- In immunohistochemical evaluation of S 100 , out of 55 cases 47 cases showed thickened positivity, 6 cases showed focal positivity and 2 cases were S 100 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 ($p < 0.001$)
- 100% sensitive but specificity cannot be arrived since it needs larger samples.If 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 as long segment and total aganglionosis had a very grave prognosis of having high mortality.

CONCLUSION

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. Serial section of biopsy should always be done for interpretation. Minimum of 20 sections should be given for confirming the diagnosis of Hirschsprung disease.

Immunohistochemistry can be used when there is difficulty and strong suspicion of 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 a valid significant results it should be studied extensively with larger big sample size . Since toluidine blue special stain is cheap ,easily available and not time consuming it can be used as an alternative for immunohistochemistry.

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MASTER CHART

S.NO	BIOPSY NO	AGE	SEX	CLINICAL DIAGNOSIS	PROCEDURE DONE	SPECIMEN	IMAGING FINDINGS	HPE- ganglion cells	hypertrophic nerve trunk	CALRETININ	S 100	TOLUIDINE BLUE
1	903/18	3/365	M	?HD	COLOSTOMY	SIGMOID COLON	DILATED BOWEL LOOPS	Absence of ganglion cell	absent	positive	focal positive	positive
2	796/18	2/365	M	hirschprung disease	biopsy	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
3	795/18	4	M	?HD	PULL THROUGH		DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
4	760/18	3/265	M	?HD	BIOPSY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	absent	positive	focal positive	positive
5	763/18	5 MONTH	M	RECTOSIGMOID HD	BIOPSY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
6	697/18	3	M	RECTOSIGMOID HD	BIOPSY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
7	601/18	15 MONTHS	M	HD	PULL THROUGH		DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
8	561/18	3/365	M	?HD	BIOPSY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
9	469/18	4 1/2	M	?HD	BIOPSY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
10	447/18	0	m	?HD	BIOPSY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
11	414/18	2/365	M	RECTOSIGMOID HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
12	366/18	2 1/2	M	HD	SIGMOID COLOSTOMY	PROXIMAL STOMA	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
13	267/18	4/365	M	RECTOSIGMOID HD	COLOSTOMY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
14	266/18	3/365	M	distal sigmoid HD	COLOSTOMY	rectal biopsy	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
15	252/18	4	M	HD	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative

16	182/18	6 MONTHS	M	HD	COLOSTOMY	DISTAL END OF COLOSTOMY	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
17	142/18	12 MONTHS	M	LONG SEGMENT HD	DUHAMEL	RECTUM AND SIGMOID	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
18	121/18	28/365	M	RECTOSIGMOID HD	COLOSTOMY	NARROW SEGMENT	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
19	63/18	3/365	F	LONG SEGMENT HD	COLOSTOMY	DISTAL LIMB COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
20	30/18	10/365	M	RECTOSIGMOID HD	COLOSTOMY	SEROMUSCULAR BIOPSY	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
21	1295/17	24/365	M	?HD	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	absent	negative	negative	negative
22	1294/17	21/365	M	?HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
23	1186/17	3/365	M	?HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
24	1173/17	6/365	m	?HD	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
25	1080/17	9/365	M	HD	COLOSTOMY	NARROW SEGMENT	DILATED BOWEL LOOPS	Absence of ganglion cell	absent	negative	focal positive	negative
26	1076/17	2/365	M	RECTOSIGMOID HD	COLOSTOMY	DISTAL NARROW SEGMENT	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
27	1031/17	4/365	M	RECTOSIGMOID HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
28	1005/17	5/365	M	hirschprung disease	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
29	1004/17	5/365	M	TOTAL aganglionosis	ileostomy	SIGMOID COLON	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
30	1003/17	4/365	M	hirschprung disease	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
31	952/17	5/365	M	DISTAL SIGMOID COLON HD ITH ILEAL PERFORATION	COLOSTOMY	DISTAL SIGMOID COLON	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative

32	892/17	5/365	M	RECTOSIGMOID HD	COLOSTOMY	DISTAL STOMA	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
33	780/17	2/365	F	HD UPTO SPLENIC FLEXURE	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
34	769/17	16/365	M	HD	COLOSTOMY	PERITONEAL REFLECTION	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
35	713/17	4/365	M	HD	COLOSTOMY	RECTOSIGMOID SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
36	390/17	2 months	F	HD	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	absent	positive	negative	positive
37	368/17	4/365	F	HD	COLOSTOMY	COLOSTOMY SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
38	254/17	2/365	M	HD	COLOSTOMY	PERITONEAL REFLECTION	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
39	229/17	4 YEARS	M	HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
40	154/17	10/365	M	?HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
41	50/17	4/365	F	LONG SEGMENT HD	COLOSTOMY	TRANSITION ZONE	DILATED BOWEL LOOPS	Absence of ganglion cell	absent	negative	focal positive	negative
42	32/17	9 MONTHS	M	HD	DUHAMEL	NARROW SEGMENT	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
43	1228/16	9 MONTHS	M	?HD	COLOSTOMY	RECTOSIGMOID SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
44	1216/16	2 YEARS	M	?HD	COLOSTOMY	RECTOSIGMOID SITE	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
45	1200/16	18 MONTHS	M	TOTAL aganglionosis	COLOSTOMY	SIGMOID COLON	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
46	1006/16	18/365	M	RECTOSIGMOID HD	COLOSTOMY	PROXIMAL STOMA	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative
47	966/16	2 years	M	RECTOSIGMOID HD	COLOSTOMY	PROXIMAL STOMA	DILATED BOWEL LOOPS	Absence of ganglion cell	present	negative	positive	negative

48	**938/16**	**30/365**	**FCH**	**PERFORATED BOWEL SEGMENTS**	**COLOSTOMY**	**RECTOSIGMOID SITE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**absent**	**negative**	**focal positive**	**negative**
49	**923/16**	**3/365**	**M**	**RECTOSIGMOID HD**	**COLOSTOMY**	**TRANSITION ZONE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**present**	**negative**	**positive**	**negative**
50	**914/16**	**4 MONTHS**	**M**	**RECTOSIGMOID HD**	**COLOSTOMY**	**TRANSITION ZONE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**present**	**negative**	**positive**	**negative**
51	**879/16**	**7/365**	**M**	**RECTOSIGMOID HD**	**COLOSTOMY**	**TRANSITION ZONE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**present**	**negative**	**positive**	**negative**
52	**834/16**	**4 1/2 YEARS**	**M**	**HD**	**DUHAMEL**	**COLOSTOMY STOMA**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**absent**	**negative**	**focal positive**	**negative**
53	**817/16**	**2/365**	**M**	**HD**	**COLOSTOMY**	**TRANSITION ZONE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**present**	**negative**	**positive**	**negative**
54	**786/16**	**1 YEAR**	**M**	**HD**	**COLOSTOMY**	**RECTOSIGMOID SITE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**present**	**negative**	**positive**	**negative**
55	**778/16**	**1 YEAR**	**M**	**HD**	**COLOSTOMY**	**RECTOSIGMOID SITE**	**DILATED BOWEL LOOPS**	**Absence of ganglion cell**	**present**	**negative**	**positive**	**negative**