COMPARISON OF MICROBIOTA OF THROAT IN CHILDREN WITH RECURRENT TONSILLITIS VERSUS ASYMPTOMATIC CHILDREN- A PILOT STUDY

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF M.S BRANCH IV OTORHINOLARYNGOLOGY EXAMINATION OF THE TAMIL NADU DR. M.G.R.MEDICAL UNIVERSITY TO BE HELD IN APRIL 2017.

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DEPARTMENT OF OTORHINOLARYNGOLOGY
CHRISTIAN MEDICAL COLLEGE
VELLORE

CERTIFICATE

This is to certify that the dissertation entitled "**Comparison of microbiota of throat in children with recurrent tonsillitis versus asymptomatic children- a pilot study**'' is a bonafide original work of **Dr. Chinnu Ann Stephen**, submitted in partial fulfilment of the rules and regulations for the M S Branch IV, Otorhinolaryngology examination of The Tamil Nadu Dr. M.G.R. Medical University to be held in April 2017.

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I declare that this dissertation entitled "**Comparison of microbiota of throat in children with recurrent tonsillitis versus asymptomatic children- a pilot study**'' submitted towards fulfilment of the requirements of the Tamil Nadu Dr. M.G.R. Medical University for the MS Branch IV, Otorhinolaryngology examination to be conducted in April 2017, is the bonafide work of Dr. Chinnu Ann Stephen, postgraduate student in the Department of Otorhinolaryngology, Christian Medical College, Vellore.

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1. IRB Application format
2. Proforma
3. Information Sheet and Informed Consent Form (English , Tamil, Hindi, Telugu)
4. Cvs of Drs. Chinnu Ann Stephen, Mary John, Ajoy M Varghese, Naina Picardo, Shalini Anandan, V. Balaji, John Jude
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We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawa for the study entitled: "Comparison of the microbiota of throat in children with recurrent tonsillitis vc asymptomatic children" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

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A sum of 93,925/- INR (Ninety Three Thousand Nine hundred and twenty five Only) will be granted for 2 years. 50,000/- INR (Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 43,925/- INR (Forty Three Thousand Nine hundred and twenty five only) will be released at the end of the first year as 2 nd Installment and out of which a maximum of Rs.5000/- can be spent for stationery, printing, Xeroxing and computer charges(if computers used are within the institution)

Yours sincerely

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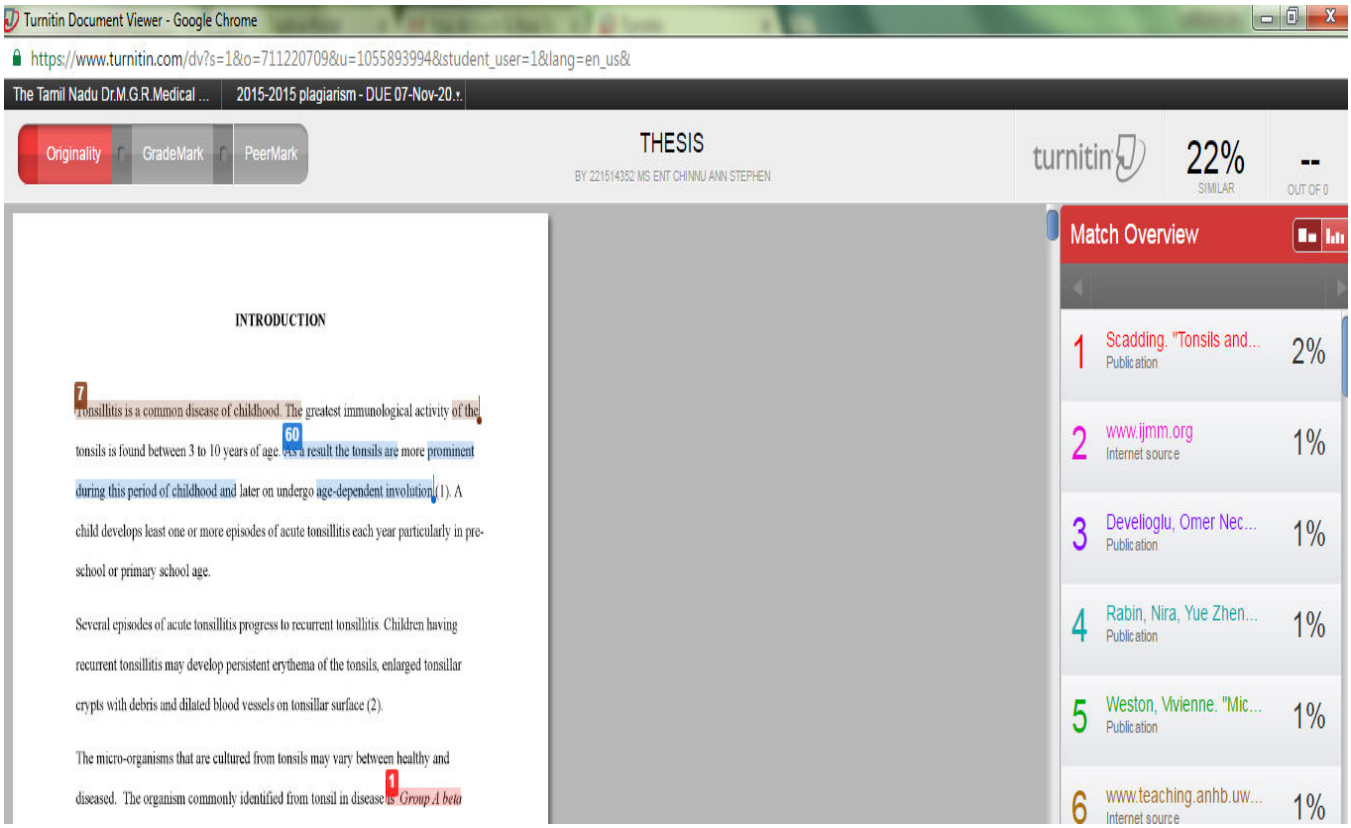
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INTRODUCTION
Tonsillitis is a common disease of childhood. The greatest immunological activity of the tonsils is found between 3 to 10 years of age. As a result the tonsils are more prominent during this period of childhood and later on undergo age-dependent involution (1). A child develops least one or more episodes of acute tonsillitis each year particularly in pre-school or primary school age.
Several episodes of acute tonsillitis progress to recurrent tonsillitis. Children having recurrent tonsillitis may develop persistent erythema of the tonsils, enlarged tonsillar crypts with debris and dilated blood vessels on tonsillar surface (2).
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I am grateful to all the ENT operation theatre staff for assisting me in collecting samples for the study and to the patients who consented to be a part of this study.

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Above all, my heart rises in gratitude to the Lord Almighty for all his blessings, throughout my life and especially during this study.

Contents

INTRODUCTION

Tonsillitis is a common disease of childhood. The greatest immunological activity of the tonsils is found between 3 to 10 years of age. As a result the tonsils are more prominent during this period of childhood and later on undergo age-dependent involution (1). A child develops least one or more episodes of acute tonsillitis each year particularly in pre-school or primary school age.

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The micro-organisms that are cultured from tonsils may vary between healthy and diseased. The organism commonly identified from tonsil in disease is *Group A beta hemolytic streptococcus* (GABHS) .But upto 40% of non-symptomatic individuals may also have a positive culture for this organism (3). Acute GABHS infections affect the susceptible populations, especially young children. It can cause life-threatening non-suppurative sequelae like rheumatic fever and acute glomerulonephritis(4).

There is a dynamic interaction of competition and regulation between potentially pathogenic bacteria and commensals in the upper respiratory tract. It is an inverse relation with pathological bacteria and commensal flora. The tonsil of children with history of recurrent GABHS infection contain less aerobic and anaerobic bacteria that can interfere with growth GABHS when

compared to asymptomatic children (5). This suggests that the presence of interfering bacteria may play a role in preventing infection.

A bacterial biofilm is a group of microbes enclosed in a self-produced matrix composed of polysaccharides, nucleic acid and proteins. In a study by Galli et al, they found high percentage of biofilm identification (100% in adenoids and 60% in tonsils samples). They came to a conclusion that biofilms are responsible for the reactivation of chronic infection .Biofilms can also cause continual tissue damage (6).

Thus better understanding of the microbiological profile of tonsils in symptomatic and asymptomatic groups of children, especially the commensal bacteria and biofilm forming pathogenic bacteria is relevant and is the broad theme of this study.

AIMS AND OBJECTIVES

Aim

The aim of the study is to compare the microbiological profile of the throat, in children less than 14 years, with or without throat infection.

Objectives

The objectives of the study are:

1. Describe the microbiological profile of throat in children with recurrent throat infection and children who are asymptomatic.

2. To quantify the commensal flora in the two groups.

3. To determine the biofilm forming capacity of the symptomatic group.

REVIEW OF LITERATURE

Epidemiology

One of the common pathogen causing tonsillitis is Group A beta hemolytic streptococcus. GABHS can cause life threatening infections like Rheumatic fever and glomerulonephritis. Hence early diagnosis and treatment of throat infection is very important.

There is no accurate estimate of the global burden of disease caused by GABHS .Carapetis et al, roughly estimated that there are around 507,000 deaths each year due to sequelae of GABHS diseases. The prevalence of severe GABHS disease is around 18.01 million cases, with 1.70 million new cases each year. The greatest burden is due to rheumatic heart disease (7).

Anatomy of oropharynx including tonsils

Oropharynx extends from level of hard palate above to the level of hyoid below.

Oropharynx is bounded by retropharyngeal space posteriorly and lies opposite the second vertebrae .Anteriorly, it communicates with oral cavity directly above , but below it is bounded by : valleculae ,base of tongue and lingual tonsil. The lateral border is mainly formed by tonsils.

Tonsils are subepithelial lymphoid collection within the triangular fossa (sinus) between the anterior palatoglossal pillar and posterior palatopharyngeal pillar (8).

The lateral surface of each tonsil is covered by pharyngeal fascia and is attached to the superior pharyngeal constrictor muscle. Condensations of the fascia form a capsule. From this capsule, trabeculae extend into the tonsil tissue and support nerves, blood vessels and efferent lymphatic vessels. The medial surface of the tonsil is covered by stratified squamous epithelium that extends into the tonsil tissue forming blind pouches or crypts. The epithelium lining the crypts is thin and allows sampling ingested material . The crypts extend deep into the substance of the tonsil and come into contact with the lymphatic germinating follicles.The crypts are usually 8 to 10 in number. With swelling of the tonsil like in infections the bottom of the crypts remains relatively fixed and the crypts become longer. The germinating follicles are centers form young lymphoid cells. The interfollicular tissue has ymphoid cells in various stages of development (1).

Tonsillar disease can reach other related anatomical structures like the Eustachian tube-middle ear complex ,paranasal sinus and upper aerodigestive tract (10).

Blood supply of tonsil

The arterial blood supply of the tonsil are typically three arteries at the lower pole: the tonsillar branch of the dorsal lingual artery anteriorly, the ascending palatine artery of the facial artery posteriorly, and the tonsillar branch of the facial artery entering between these two in the lower aspect of the tonsillar bed (11). At the upper pole of the tonsil the ascending pharyngeal artery enters posteriorly and the lesser palatine artery enters on the anterior surface. Out of the three main supplies, the tonsillar branch of the facial artery is the largest. Venous blood drains to the peritonsillar plexus around the capsule (11).The plexus drains into the lingual and pharyngeal veins. This in turn drain into the internal jugular vein (12).

Nerve supply of tonsil

The nerve supply of the tonsillar region is through the tonsillar branches of the glossopharyngeal nerve and through the descending branches of the lesser palatine nerves. The cause of referred otalgia with tonsillitis is through the tympanic branch of the glossopharyngeal nerve (12).

Lymphatics of tonsil

The foreign bodies entering oropharynx is exposed to lymphoid tissue through tonsillar crypts. Tonsils does not have afferent lymphatic drainage. Efferent lymphatic drainage courses through the upper deep cervical lymph nodes, especially the jugulodigastric or tonsillar node located behind the angle of the mandible (12).

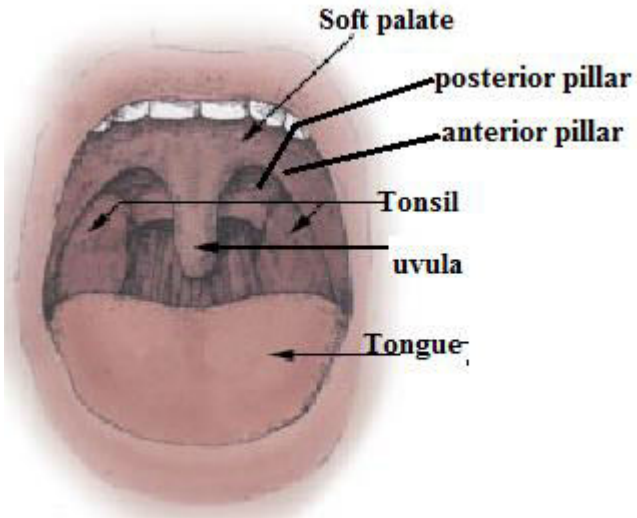


Figure 1: Anatomy of oropharynx

Immunology of tonsils

Tonsils are part of the Waldeyer's ring. Waldeyer's ring is a "ring" of lymphoid tissue, which provides the first contact of ingested or inhaled pathogens with the lymphoid system. It is composed of the (1) adenoid, (2) palatine tonsils, (3) lingual tonsil, (4)

scattered lymphoid follicles, (5) lateral pharyngeal bands, and (6) nodules near the Eustachian tube. This lymphoid tissue has only efferent lymphatics. It is located to sample all substances that enter the aerodigestive tract. Waldeyer's ring is involved in the development of B cells, especially in the first few years of life. It also produces a major classes of immunoglobulins and T lymphocytes involved in cell mediated or "delayed" immunity (2).

The tonsils and adenoids are predominately B-cell organ: B lymphocytes comprise 50 to 65% of all adenoid and tonsillar lymphocytes (13). The immunoreactive lymphoid cells of the adenoids and tonsils are found in four areas: the mantle zone of the lymphoid follicle, the reticular cell epithelium, the germinal center of the lymphoid follicleand the extrafollicular area and (12).

Of the different components that form the Waldeyer's ring, the tonsils and adenoids play the major role in defense mechanism. Intratonsillar defense mechanisms eliminate weak antigenic signals. Only when higher antigenic concentrations are presented does proliferation of antigen-sensitive B cells occur in the germinal centers (13). Low antigen doses effect the differentiation of lymphocytes to plasma cells whereas high antigen doses produce B-cell proliferation. The generation of B cells in the germinal centers of the tonsils is considered by Siegel as one of the most essential tonsillar functions (12).

But in recurrent tonsillitis, inflammation of the crypt epithelium causes shedding of immunologically active cells and decreased antigen transport function and subsequent replacement of germinal centers by stratified squamous epithelium(15)(16) .These changes lead to decreased activation of the local B-cell system and overall decrease in density of the B-cell and germinal centers in extrafollicular areas (12).
The micorganisms responsible for infection of middle ear first proliferate in the oropharynx or nasopharynx. Thus the tonsils and adenoids play a significant role in preventing otitis media (17).

Histopathology of tonsils

The tonsils have efferent lymph vessels but lack afferent lymph vessels. Exposure to antigens depends on the contact of antigens with cells of the immune system across the epithelium which covers the tonsils. The epithelium of the palatine and lingual tonsils forms deep crypts into the lymphoid tissue, and thus the resulting increase of the surface area facilitates the contact of antigens with the immune cells.

Tonsillar lymphoid nodules consist mainly of B-lymphocytes. Other areas are occupied by T-lymphocytes, activated B-lymphocytes and other cells of the immune system.

Tonsils and lymphoid tissue varies in many ways. Some similarities between the in histological features are:

1. Cells in the tonsils are supported by reticular fibers .

2. Post capillary venules functions in bringing circulating lymphocytes to all lymphoid tissues and organs.

The palatine tonsils are covered by a thick capsule of connective tissue which facilitates the easy removal of tonsils during tonsillectomy (18).

Tonsil is covered by stratified squamous epithelium on its medial surface. Cells of the immune system often invade the crypts through the reticulated epithelium near the crypts (6).

In chronic tonsillitis, there is change in the histologic features of tonsil. Microscopically there is excessive increase of the germinative center and the decrease of the follicular cortical. But the capsule ,covering epithelium and interlobular septae have about normal structure (19).

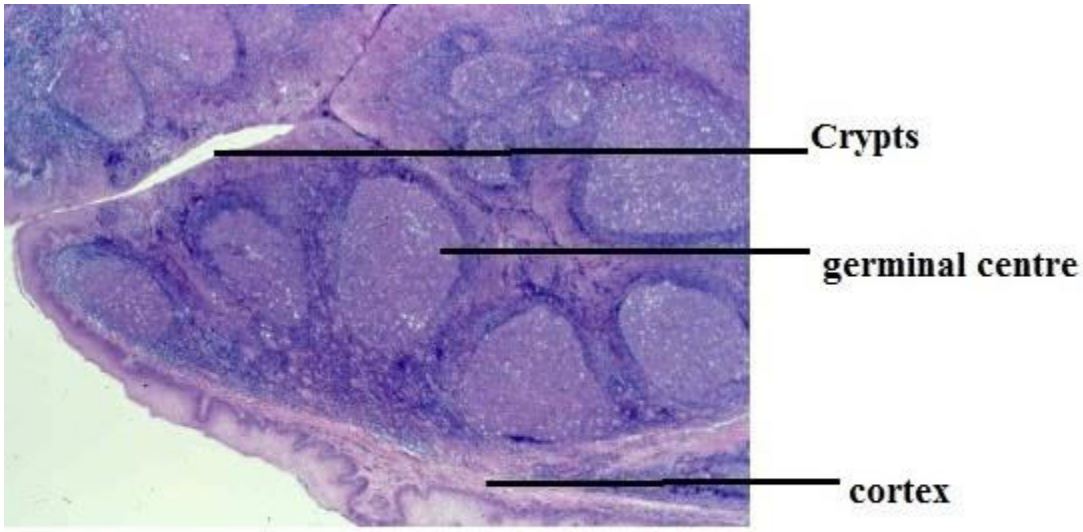


Figure 2: Histopathology of tonsil tissue

Adenoids

Adenoids are located in the nasopharynx. Adenoid tissue is present at birth.It undergoes physiological enlargement upto 6 years of age and then tends to atrophy at puberty. It almost completely disappear by 20 years of age (20). Adenoids differ from tonsil of the fact that, it is composed of vertical ridges of lymphoid tissue separated by deep clefts. The surface is covered by ciliated columnar epithelium. Adenoids have neither crypts nor capsule.

Adenoids are visualized clinically by posterior rhinoscopy. But children are usually not co-operative for posterior rhinoscopic examination. The two investigations used to grade adenoid hypertrophy are x-ray nasopharynx lateral view and rigid nasal endoscopy.

Diseases of tonsils

Disease of tonsil is also more common in young age group. The tonsil surface has crypts that extend into the parenchyma of tonsil forming pits or crypts. Microorganisms, food debris and desquamated epithelium are frequently present within the crypts and this leads to acute and recurrent inflammation and hence infection (22).

Tonsillitis refers to inflammation of the pharyngeal tonsils. The inflammation may involve other areas including the adenoids and the lingual tonsils as they all form part of Waldeyer's ring. There are several variations of tonsillitis: acute, recurrent, and chronic

tonsillitis and peritonsillar abscess (23). Viral or bacterial infections and immunologic factors lead to tonsillitis and its complications.

Acute tonsillitis

Children have fever, sore throat, dysphagia, foul breath, odynophagia and tender cervical lymph nodes.

Airway obstruction due to swollen tonsils may cause snoring or sleep apnea.These symptoms usually resolve in three to four days but can also last up to two weeks despite medical treatment.

Acute tonsillitis may be either

a) superficial or catarrhal

b) follicular or cryptic

c) membranous

d) parenchymatous(24).

Acute Superficial Tonsillitis: It involves mainly the mucous membrane covering the tonsil, but can involve deeper structures also. The process may also spread to contiguous structures .It can either undergo resolution or in case of secondary infection leads to suppuration of the connective tissue causing peritonsillar abscess.

The tonsils appear reddish, edematous and swollen. The crypts usually becomes filled with fibrin, leucocytes and epithelial debris, which forms acute follicular tonsillitis.

***Acute follicullar tonsillitis*:** Acute follicular or cryptic tonsillitis, is an acute infection of the tonsils. There is necrosis of the mucous membrane covering the tonsils, together with

fibrin exudates leading to the formation of irregular patches of pseudo-membrane. It is known as “pseudo-diphtheria”.

Tonsils are deeply congested and their surface is studded with yellowish-white, punctuate spots that appears at mouth of the crypts.

Acute membranous tonsillitis: Here the exudates from crypts coalesces on the surface of the tonsils and gives the appearance of a membrane. The membrane is superficial and hence can be easily wiped out, distinguishing it from diphtheria.

Acute parenchymatous tonsillitis: Here tonsillar substance is affected. Tonsil is uniformly enlarged and red (25).

Recurrent tonsillitis

This diagnosis is made when an individual has multiple episodes of acute tonsillitis .The recurrent episodes of tonsillitis is managed by tonsillectomy. Paradise et al, observed the efficacy of tonsillectomy in children with recurrent tonsillitis and came up with a criteria for tonsillectomy.

Recurrent tonsillitis is characterized by

sore throat – 7 or more episodes in the preceeding year,

OR 5 or more episodes in the preceeding 2 years,

OR 3 or more episodes in the preceeding 3 years (23).

The clinical features (sore throat plus any one or more of the following symptoms quantifies to count as an episode)-

a) temperature >38.3^{0}C,

b) OR tonsillar exudates,

c) cervical lymphadenopathy (tender lymph nodes of >2 cm),

d) OR positive culture for Group A beta hemolytic streptococcus.

Chronic tonsillitis

Individuals often have chronic sore throat, halitosis, tonsillar hypertrophy and persistently tender cervical nodes.

Evaluation of tonsillitis

An accurate diagnosis of GABHS infection is important because it is the only common form of acute pharyngotonsillitis for which antibiotic therapy is definitely indicated. Antibiotic therapy can shorten the duration of GABHS pharyngotonsillitis, reduce the transmission and prevent suppurative and nonsuppurative complications (26).

Throat culture

Throat culture is the gold standard for the laboratory diagnosis of GABHS infection (27). If the sample has been collected appropriately, GABHS growth can be observed in the culture of 90-95% of patients with typical clinical symptoms. However, the completion of throat culture tests may require as long as 24 to 48 hour.It is important to start early antibiotic therapy in life-threatening infections caused by GABHS is important. But the culture tests takes 24-48 hours to provide results and if the patient has taken antibiotic, there is possibility of getting a negative result. On the other hand, the rapid antigen tests

used to identify GABHS in throat swab samples can obtain test results within less than 15 minutes (28).

Management

General measures include pain relief with gargles or systemic analgesics. Maintenance of proper hydration is important, particularly in children. If the initial presentation suggests streptococcal infection, then antimicrobials are indicated on the grounds that:

1) Suppurative complications can be reduced

2) Rheumatic fever risk is reduced

3) Symptoms are more rapidly reduced and

4) Intrafamilial spread is reduced.

Medical treatment

Pencillin is the antibiotic of choice for the treatment of streptococcal infections, particularly GABHS. Oral and intramuscular administrations are effective.

a) Pencillin G or V,125-250mg/dose three times a day for 10 days is appropriate or

b) Benzathine pencillin G 600,000-12,000,000 IU once intramuscularly.

In patients sensitive to pencillin, erythromycin at 20-40mg/g/day for 10 days may be given (29).

Despite reports of occasional therapeutic failures with penicillin, it is still the antibiotic of choice. For patients with penicillin allergy, erythromycin is the alternate drug of choice.

Although penicillin V still remains the antibiotic of choice for most cases of acute tonsillitis, there is a shift towards using alternate antimicrobials with excellent intra-tonsillar drug concentration , beta-lactamase resistance and broader spectrum .There is an increasing support for their greater clinical efficacy. Azithromycin, has prolonged half-life and achieve higher tonsillar drug concentrations (30).

Tonsillectomy

Surgical removal of the tonsils is indicated in some cases of infections and other non-infective conditions. The indications for tonsillectomy are:

1) Recurrent episodes of acute tonsillitis (according to Paradise criteria as described previously)

2) Presence or a history of a peritonsillar abscess or of a diphtheria carriers

3) Massive tonsillar hypertrophy causing respiratory obstruction in children resulting in failure to thrive.

4) Treatment of glossopharyngeal neuralgia or in the case of enlarged styloid process (Eagle's syndrome).

5) Biopsy for tissue diagnosis

Of all indications for tonsillectomy, recurrent (chronic) tonsillitis is the commonest indication (31). There is no evidence that tonsillectomy reduces rate of rheumatic fever (32).

Tonsillectomy is contraindicated in

1) Presence of acute infection

2) Heamoglobin is less than 10 gm%
3) Overt sub mucous cleft palate
4) Bleeding disorders like purpura,leukemia, aplastic anemia
5) Poliomyelitis epidemic
6) Uncontrolled systemic illness like diabetes, cardiac diseases

Procedure

Under general anaesthesia, patient is placed in Rose's position. Tongue is retracted using Boyle-Davis mouth gag. The superior pole of one tonsil is grasped with an Allis clamp and retracted toward the midline, placing the tonsillar pillar under some tension. The mucosa over the superior pole of the tonsilis incised using a long toothed forceps at the junction of the tonsil and its muscular pillars. This incision is extended anteriorly and inferiorly towards inferior pole of the tonsil. Using tonsillar dissector, muscles of tonsillar fossa are dissected away from tonsillar capsule. The dissection is continued until the tonsil is attached only by a vascular and mucosal pedicle at the inferior pole. The loop of a wire snare is then passed around the tonsil and drawn tight at the inferior pole attachement using a slow,steady motion. When the attachment of the inferior pole of the tonsil is transected, there is usually some bleeding, which is controlled by bipolar cautery and by placing adrenaline packs in the tonsillar fossa (33).

Procedure is repeated on the other side. The packs are removed from side of first tonsillectomy, and the tonsil bed is inspected carefully. Small bleeding points are electro coagulated, and larger bleeding vessels are clamped and ligated.

Complications of tonsillitis

Children with diagonised acute GABHS tonsillitis should be treated with antibiotics to eliminate the bacteria from pharynx, to improve clinical symptoms and signs, and to reduce the transmission of bacteria and to prevent suppurative complications and acute rheumatic fever (34).

Complications of GABHS tonsillitis are broadly divided into suppurative and non suppurative. The non suppurative complications are scarlet fever, acute rheumatic fever and post- streptococcal glomerulonephritis. Suppurative complications are peritonsillar abscess, parapharyngeal abscess and retroparyngeal abscess (35).

Non- suppurative complications

Acute rheumatic fever and acute glomerulonephritis are two main non-suppurative complications of acute streptococcal infection with long term implication. Both usually occur after a brief asymptomatic period and are characterized by lesions in site remote from the site of GABHS infection. However, they differ in their clinical manifestations (29).

Acute rheumatic fever: It usually follows GABHS upper airway infection with certain serotypes. Serotypes commonly isolated in patients with acute rheumatic fever are 1, 3,5,6,18 and 24 . The risk of rheumatic valvular heart disease and its long-term implications and the need for life-long prophylaxis with pencillin are the main concerns in preventing the development of acute rheumatic fever.

Acute glomerulonephritis: It occurs after infection of either the upper respiratory tract or skin by the serotype 49, infection by nephrogenic strains, serotype 12. It is characterized by gross hematuria, edema, hypertension and renal insufficiency.

Post streptococcal reactive arthritis: This syndrome is usually follows an episode of GABHS pharyngotonsillitis. It is characterized by onset of acute arthritis, usually involves large joints. It does not respond well to aspirin or other anti-inflammatory drugs.These patients are kept under regular follow up due to a small risk of valvular heart disease (29).

Suppurative complications

Peritonsillar abscess: Is collection of pus on lateral surface between the fibrous capsule of the tonsil and superior pharyngeal constrictor muscle, pushing uvula to opposite side. The most common understanding about peritonsillar abscess is that it occurs secondary to the penetration of bacteria from the tonsillar crypts through tonsillar capsule to reach the peritonsillar space. An alternative theory as described by Passy in 1994,it is an abscess collection in Weber's salivary glands in the supratonsillar fossa causes peritonsillar abscess (34).

Symptoms are difficulty in swallowing, drooling, trismus, and fever. Assymetric peritonsillar swelling can occur, with deviation of the uvula. The treatment options are aspiration of pus, incision and drainage under local or general anesthesia. Tonsillectomy can also be planned after 6 weeks once abscess is resolved (interval tonsillectomy).

Antibiotics are necessary, pencillin or clindamycin being the best choice (34).

Retropharyngeal and Parapharyngeal abscess:

The presentation of parapharyngeal and retropharyngeal abscess varies significantly. Initially, symptoms and signs mimic an upper respiratory tract infection. In children, symptoms and signs may be more non-specific like irritability and poor feeding. There is rapid progression of symptoms and signs progressing to upper airway obstruction, causing dysphagia, neck stiffness, stridor and dyspnoea . High suspicion of the abscess from the clinical examination is confirmed usually computerized tomography scan. Surgical drainage of abscess either intraoral or external drainage through the neck must be done along with appropriate parenteral antibiotics and supportive measures (36).

Diphtheria

Diphtheria is a rare now because of active immunization. However, it should be considered as a diagnosis in among children who are inadequately immunized or not immunized at all (34). Membranes may extend outside the tonsils to larynx, soft palate and /or nasopharynx. The borders of the membrane are sharply defined and the membrane is closely adherent to the underlying tissue. For diagnosis, the membrane must be cultured.

Treatment

Both anti toxin and antibiotics are mandatory. Antibiotics chosen are either erythromycin or pencillin given parenterally or orally for 14 days. Antimicrobial therapy is required to eradicate the organism and prevent spread and cannot substitute for antitoxin. Because the condition of patients with diphtheria may deteriorate rapidly, even before a proper diagnosis of diphtheria can be made with culture results, a single dose of diphtheria antitoxin can be administered. To neutralize toxin as fast as possible, the preferred route of administration is intravenous (37).

Vincent's angina

It is an acute pseudo membranous involvement of the pharynx or tonsils. The disease is mostly secondary to a combination of fusospirocheatal organisms (mostly often *Fusobacterium nucleatum* and *Treponema vincentii*) and gram-negative anaerobic organisms (*Bacteriodes* species*, Prevotella intermedius* and *Prevotella melaninogenica*).

The disease begins acutely with pain, bad odor of the breath and gingival or tonsillar bleeding. There is necrosis, pseudomembrane formation, lymphadenopathy and excessive salivation (38).

Antibiotics most commonly used for this condition are oral Pencillin (at 500mg four times daily), Clindamycin (at 600mg three times daily) or Metronidazole (at 500mg three times daily) (38).

Infectious mononucleosis:

Infectious mononucleosis is one of the diseases caused by Epstein-Barr virus (EBV). Infectious mononucleosis arises in young adults who are not infected during childhood. Symptoms include sore throat, fever and disproportionate fatigue. In those taking ampicillin,they can develop maculopapular rash and sometimes periorbital oedema . Dysphagia occurs due to tonsillar and pharyngeal oedema. Sometimes there can be greyish exudates over the tonsillar surface. Hepatosplenomegaly and lymphadenopathy resolve within one to two weeks.EBV infection can lead to recurrent bacterial tonsillitis in a proportion of patients. These patients often fail to recover and some require tonsillectomy. The use of steroids helps to resolve tonsillar swelling (3).

Microbial flora of oropharynx

A polymicrobial flora is seen in the skin and mucous membranes of every human being shortly after birth. This bacterial population constitutes the *normal microbial flora.* The normal microbial flora is comparatively stable. They may colonise the oroparynx and exist as commensal or may harm the host by causing infectious diseases (39).

The flora of a child is fairly similar to an adult, with several exceptions. At birth, the oral cavity is sterile, but colonization with a wide variety of microbes occur within hours. Although many sources of oral microbial colonization exist, a predominant source has been shown to be the oral flora of infant's mother. *Streptococcus salivarius* has been

found in 80% of cultures taken from 1-day-old infants. The percentage of Streptococcal species decreases from 98% on the day after birth to 70% at 4 months of age. *Staphylococcus species, Neisseria, Corynebacterium ,Vellonella, Actinomyces, Norcardia, Bacteriods, Fusobacterium*, Candida and a variety of coliform gradually become established by age of 1 year. Once eruption of primary dentition occurs, anaerobic organisms become established in the gingival crevice (40).

The normal microbial flora in healthy individuals displays quantitative and qualitative stability that prevents invasion by foreign microorganism. However, the equilibrium is hampered by various factors like exposure to broad spectrum antibiotics ,changes in nutritional, hormonal or physical environment of microbial flora.

Many members of normal flora of the pharynx are neither fully identified nor reported in microbiological report even when they are observed in throat cultures (41). The most common micro organisms of normal flora are:

• *Streptococci viridans* (alpha-haemolytic) and pneumococci
• nonpathogenic *Neisseria* spp
• *Branhamella* (formerly *Neisseria catarrhalis*)
• diphtheroids
• *Haemophilus* spp
• various strictly anaerobic microbes, spirochaetes and filamentous forms.

Pathogenic organisms of oropharynx

In a study on all age group by A Agarwal et al in Agra, common causative organisms of tonsillitis isolated were *Alpha-hemolytic streptococci*, non-pathogenic *Neisseria* species, *Haemophilus influenza*, *Staphylococcus aureus*, Pneumococcus*, , Bacteriods fragilis*, Enterococcus and corynbacterium species. No anaerobes were identified (8).

In another study by Omer Necati Develioglu et al in Turkey, they observed the anerobes from surface and core of tonsils in patients with recurrent tonsillitis. The common isolated facultative anaerobic species were *Coagulase-negative staphylococci* and Diphtheroid bacilli. On the other hand, the commonest isolated obligate anaerobic species were *Prevotella melaninogenica ,Propionobacterium acnes* and *Peptostreptococcus anaerobius*(42).

Beta-hemolytic streptococci

This group of streptococci are separated from other group by their ability to produce complete haemolysis of blood when isolated on blood agar They are separated using the Lancefield group antisera. The main human isolates group with A, B, C, D (enterococci) and G antisera (3).

Group A(Streptococcus pyogenes), C and G streptococci

Group A beta hemolytic streptococcus (*Streptococcus pyogenes*) is the most common one producing the suppurative diseases of tonsillitis, skin infection and, rare but severe

infections like necrotizing fasciitis, invasive disease like septicemia and streptococcal toxic shock syndrome.

Groups C and G streptococci rarely produce disease. In healthy subjects, Groups A, C and G streptococci are found colonizing the nasopharynx.

Viridians streptococci

The oral streptococci produce incomplete haemolysis when inoculated on blood agar. They are part of the indigenous flora of oropharynx and nasopharynx They can occasionally cause local disease like dental abscesses. The major cause for native valve endocarditis is S.*viridians*. For this antibiotic prophylaxis is given during dental and surgical procedures. Some species of oral streptococci have been found colonization the nasopharynx and inhibit growth of pathogenic bacteria. Hence they are used in clinical trials as probiotics.

Non pathogenic Neisseria

Most *Neisseria* spp. are Gram-negative cocci that commonly occurs in pairs (diplococcic), tetrads or short chains (43). The diplococcic have their adjacent sides flattened, giving them the appearance of side-by-side coffee beans or kidneys. All *Neisseria* spp share the following characteristics:

- Inhabit mucosal surfaces of warm-blooded hosts
- Aerobic
- Nonmotile
- Non-spore forming
- Optimal growth temperature at 35^{o}C to 37^{o}C.
- Produce acid from carbohydrates by oxidation, rather than by fermentation.
- Oxidase positive
- Most species are catalase positive.

Pathogenic organisms tend to be more fastidious than non-pathogenic species. They are very demanding in nutrients and their growth is stimulated by CO_2 and humidity. The following *Neisseria* spp. may be isolated from human specimens:

- *N.gonorrhoea :* the causative organism of gonorrhea, always regarded as pathogen regardless of the site from where it is isolated.
- *N.meningitidis*: a significant pathogen, but may also colonise, the human nasopharynx without causing disease.
- *Non pathogenic Neisseria (N. cinerrea, N.elonga ,N.flavescens ,N.kochii, N.lactamica,N.mucosa,N.polysaccharea flava and N.sicca, N.subflava)*

With the exception of *N.gonorrhoea* and *N.meningitidis,* most *Neisseria* spp form commensals of the upper respiratory tract and are nonpathogenic. Different methods are used to differentiate *Neisseria* spp. including carbohydrate utilization, colorimetry

involving chromogenic substrates, immunodiagnostic procedures using monoclonal antibodies, isoenzyme electrophoresis and DNA probes.

Moraxella (Branhamella) cattarhalis

Moraxella (Branhamella) cattarhalis, is an aerobic oxidase positive, non-motile gram negative diplococcus. Colonies on blood agar are non-hemolytic forming grayish white colonies with each organism having round, opaque and convex appearance (44).

M. catarrhalis was considered as simple colonization of the upper airway . Additionally, it is difficult to detect *M. catarrhalis* on a primary isolation plate of a respiratory sample where other oral flora or pathogens are also present.

The main features that distinguish *M. catarrhalis* from other bacterial species are: DNase production; does not produce acid from glucose, maltose, sucrose, lactose, fructose; failure to thrive in modified Thayer-Martin(MTM) medium; and ability for reduction of nitrite and nitrate.

Diphteroids

Other *Corynebacterium* species form indigenous flora of skin, throat conjunctiva and other areas. They resemble C.diphtheriae and may sometimes be mistaken for diphtheria bacilli and are called diphteroids or nondiptherial corynebacteria (45).

Features to differentiate between corynebacterium diphtheria and diphtheroids

Characteristics	C. *diphtheria*	Diphtheroids
Metachromatic granules	present	Few or absent
Staining property	Uniformly stained	More uniformly stained
Arrangement	Cuneiform	Parallel rows
Culture	Grows on enriched medium	Grows on ordinary medium
Sugar fermentation	negative	Positive
Toxins	Produces toxins	doesnot produce toxins with few exceptions(eg:C.*ulcerans*,C.*pseuotuberculosis*)

Haemophilus spp

The Genus *Haemophilus* include *H.influenzae, H.parainfluenzae, H.ducreyi ,H.aegyptius, H.paraphrophilus ,H.aphrophilus, H.heamolyticus, , and H.segnis.*

H.influenzae is a small (0.2-0.3x0.5-0.8micrometer) Gram-negative nonmotile coccobacillus that grows well on rich media usually, chocolate agar medium incubated in

5% CO2 (46). It has the following specific growth requirements: it grows only on nutrient agar with growth supplements X factor (haemin) and V factor(NAD). *H.influenzae* produces 2 to 3 mm diameter grey translucent colonies after 18 to 25 hr incubation.

H.influenzae has various cell surface features essential for colonization of the nasopharynx. These are virulence factors out of which capsule are most important. There are six antigenically distinct structures of *H.influenzae* namely (types a-f), out of which type b accounts for virtually all the invasiveness of *H.influenzae* in children. The serotype b capsule consists of a negatively charged polymers of disaccharide units of polyribosylribitol phosphate (PRP) linked by phosphor diester bond. This resists phagocytosis by interfering with binding of serum complement. The capsule also resists dessication, perhaps promoting host-to-host transmission. Serum antibody directed against serotype b capsular polysaccharide is protective. This principle was used in the development of the highly successful *H.influenzae* type b (Hib) vaccine, routinely used in national childhood immunization programmes.

Haemophilus parainfluenza

H.parainfluenza is a commensal that colonises everyone soon after birth but is rarely associated with disease. Unlike, *H.influenzae* it requires only factor V for its growth. It is reported to cause sub acute bacterial endocarditis,urethritis and acute pharyngitis (47). It is treated in the same way as *H.influenzae.*

Anaerobes

Anaerobes are abundant among the normal flora of the oropharynx. Some anaerobic bacteria are capable of inhibiting the growth of GABHS as well as other potential pathogens causing tonsillitis (5).

Various factors that modify the microbial population including age, anatomical relationships, eruption of teeth, presence of decayed teeth, diet, oral hygiene, antibiotic therapy, any systemic diseases and hospitalization. The estimated ratio of anaerobic to aerobic organisms in the oral cavity ranges from 3:1 to 10:1 (40).

The anaerobic species that cause tonsillitis are pigmented *Prevotella* and *Porphyromonas, Fusobacterium and Actinomyces* spp.(48).

In a study by Brooke et al, they found same polymicrobial aerobic and anaerobic bacterial flora in the core and surface of recurrently inflamed tonsils. But the number of species of organism and concentration of bacteria was much higher in children with recurrently inflamed tonsils (48).

Prevotella* and *Porphyromonas

Both *Prevotella* and *Porphyromonas* were previously classified under the genus *Bacteriodes*. Now they are grouped under, Gram negative anaerobic organisms. These pigmented Gram negative anaerobes can be divided as based on their characteristics of metabolism as the saccharolytic *Prevotella* spp and the assaccharolytic *Porphyromonas* spp. Approximately 20 of the *Prevotella* spp have been implicated in causing human disease. *Prevotella* forms circular, convex 1 to 2 mm,shiny, gray colonies. On gram stain

they form short gram-negative rods and assume coccobacilli forms. *Prevotella,* grows well on laked blood agar with kanamycin and vancomycin (LKV) and has shown variable resistance to collistin. Although, *Prevotella,*spp are largely considered as pigmented Gram negative anaerobes, some species are nonpigmented as well. Pigmented species form brown or black colonies after a week of growth on LKV.

Porphyromonas spp , though tend to form smaller colonies and present as shorter rods or coccobacilli on Gram stain but can be difficult to distinguish from *Prevotella.*

Porphyromonas usually grow as pigmented colonies,that initially form gray colonies and later darken to black colonies within a week after plating on laked blood agar.

Porphyromonas does not grow on LKV media because they are sensitive to vancomycin, but its resistance to collistin (49).

Fusobacterium

Fusobacterium is a genus of obligately anaerobic filamentous gram-negative rods that form members of phylum Fusobacterium (49). *Fusobacterium* is highly virulent anaerobe with 20 species. A main component of cell wall is a lipopolysaccaride (LPS) .Though both *Fusobacterium* and *Bacteriodes* has LPS, the difference between the two is that the *Fusobacterium* LPS molecules secrete an endotoxin while the *Bacteriodes* molecules do not (50). Depending on the strain, Fusobacterium can be hemolytic. *Fusobacterium* spp can be somewhat variable in their Gram stain and display a wide range of cellular morphologies from being coccoid, pleomorphic sperules (*Fusobacterium necroporum)* to being rod shaped.

Fusobacterium is sensitive to both kanamycin and collistin and resistant to vancomycin. It can be distinguished by its sensitivity to bile and conversion of threonine to propionate. Most species are indole positive and on fermentation of glucose produce butyric acid (49).

Actinomyces

Actinomyces are facultative anaerobic, gram stain positive bacteria constitute 15 to 29% flora in saliva and tongue. There are about 24 species of Actinomyces. Although *Actinomyces* spp are fastidious , several complex general culture media have been found to isolate these organisms from the oral cavity of humans (51).

Most of the commensal flora overgrow the *Actinomyces* in culture media. Hence their growth in culture media are overlooked or even suppressed.

Actinomyces like organisms are found in chronically inflamed tonsils but their exact role in tonsil infection is unclear (52).

Anaerobes as interfering bacteria

Anaerobic organisms found in the indigenous flora prevent colonization and growth of pathogenic organisms. This is known as bacterial interference. This phenomenon prevents certain bacterial infections.

The children with the history of recurrent GABHS infection was found to have less anaerobic bacteria in their tonsils when compared to those children without history of GABHS infection. In other words, it means that the presence of these bacteria interferes with growth of GABHS (48).

Probiotics

The upper respiratory tract is harbours a lot of commensal organisms. Commensal bacteria act as natural competitors of pathogens .They adhere to binding sites on epithelial cells.Inappropriate use of antibiotics could remove this competition, leading to colonization of upper respiratory tract with more virulent and antibiotic-resistant pathogenic strains (53).

Probiotics are live microorganisms that offer health benefits by modulating the microbial community and enhancing host immunity (54).

The main aim of probiotic treatment is to modify the composition of the microbiota from potentially harming to a benefical microbiota in the host. Probiotics can be used as a way of prevention by suppressing colonization of pathogenic bacteria .They also help to restore the lost bacteria or metabolic activities in colonized anatomical sites or to stimulate the immune response (55).

The concept of using alpha- hemolytic streptococci as probiotics was attempted by Sprunt et al (Sprunt and Redman in 1968 and Sprunt and Leiy in1988). In one of the studies conducted by them,they used a strain of *alpha-hemolytic streptococcus* and inoculated it into the nasopharynx of neonates admitted in the intensive care unit. They found that with a single inoculation alpha-streptococci became predominant in nasopharynx within 48-72 hours in most neonates replacing pathogenic strains. They also found that *Streptococcus* with same characteristics like the strain used in the study was

found naturally in 17% of neonates in that particular intensive care unit. This study emphasized the concept of probiotics (56).

Anaerobes as pathogen

The role of anaerobic bacteria in causing tonsillitis is not known, but there is increasing evidence that anaerobes as a cause of tonsillitis cannot be underestimated. One of the anaerobic infection of tonsils is Vincent's angina, which can be diagnosed by taking a scraping from the ulcerative tonsil (or gingiva). Vincent's angina is treated with metronidazole and pencillin. Another example of anaerobic bacteria causing chronic or recurrent tonsillitis is *Fusobacterium necrophorum*, which is best eradicate with clindamycin or metronidazole (34).

Effect of antibiotics on oropharyngeal flora

Eventhough there is variation from person to person, the composition and the number of different microorganisms in the normal microflora remain about the same. Microflora can change when physiological or ecological disturbances occur in the upper respiratory tract. Any disturbance in the environment of normal flora like extraction of the teeth or irradiation of the head and neck causes marked changes in the microflora. The most common cause of disturbance in the normal human microflora is the use of antibiotics. Though antibiotics are useful in infections to destroy the pathogens. Its inappropriate use can suppresss the commensal flora . Antibiotics that influence the normal microflora also contribute to the emergence of antibiotic-resistant strains (57).

The use of antibiotics always precedes tonsillectomy. However, repeated use of antibiotics can lead to antibiotic-resistance and may not eradicate pathogenic bacteria from the tonsils. This is the reason why though, penicillin was initially the treatment of choice for tonsillitis, its use has increased failure rates to 20 % (58).

Other causes for antibiotic resistance are fibrosis of tonsils as a result of recurrent tonsillitis. This can prevent penetration of the antibiotic into the tonsil tissue. Another explanation is the presence of bacterial biofilm around the tonsil tissue. In a study by Woo et al. (59) tonsillar biofilms were significantly prevalent and were found at higher grade in the recurrent tonsillitis group than in the control group (60).

Biofilm bacteria are particularly resistant to antibiotic treatments because of a number of reasons. It may be due to increased transmission of resistance markers within the biofilm community, decreased diffusion of antibiotics caused by the extracellular matrix and the presence of high concentration of metal ion and low pH. Another cause of resistance is the presence of metabolically inactive cells that survive treatment .All these factors of biofilm make the bacteria up to 1000-fold more tolerant and resistant to antibiotics. Thus there is a need for more effective treatment against biofilm (61).

Microbial evaluation using throat culture

According to microbiology reporting protocol, a micro- organism in heavy growth in oropharynx can be colonizer or a pathogen and the microbiology report has to be correlated with clinical symptoms and signs. Hence, a micro-organism in heavy quantity in throat that causes tonsillitis is considered as pathogen. On the other hand, an organism in heavy

quantity in oropharynx in asymptomatic group is considered as a coloniser. Hence *H.parainfluenza, Group B hemolytic streptococci, Streptococcus pneumonia, H.influenza, Group G beta hemolytic streptococci, Group A beta hemolytic streptococci, Pseudomonas aeuroginosa, Group F beta hemolytic streptococci, Klebsiella* and *Staphylococcus aureus* are the micro-organisms cultured from throat swab. These organisms in scanty or moderate quantity are considered as commensal flora. When in heavy quantity, they are considered as a pathogen or colonizer depending upon the clinical symptoms. Only *Alpha hemolytic streptococci, non pathogenic Neisseria, and non- fermenting Gram negative bacilli* are purely commensal organisms.

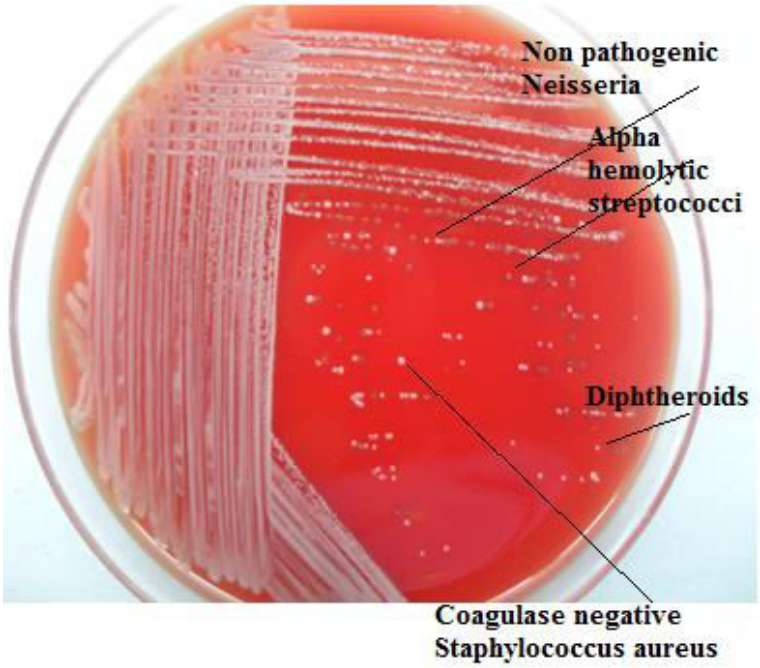


Figure 3: Culture plate showing growth of commensals

Biofilm

A biofilm is defined as a sessile microbial community characterized by adhesion to a solid surface by production of a matrix, which surrounds the bacterial cells and includes extracellular polysaccharides (EPS), proteins and DNA (62).So it is an assembly of microbial cells that is well protected within a self-produced polymeric matrix. The composition of matrix differs according to the surface in which the bacterial cells grow.

Antonie van Leeuwenhoek (1632-1723) was the first person to bring out the concept of microorganisms that binds to a surface in the form of dental plaque (63).He used a simple light microscope, to describe the same. However, the concept of bacterial biofilm reemerged many years later in 1970s.This was noted by Characklis (64).

It is now believed that a bacterias in biofilm do not exist singularly as planktonic organisms but rather as well-organized ecosystems. These complex ecosystems are well are adapted for conditions of environmental stress (65).

Biofilms are tough and shield organisms from disinfectants and even ultraviolet radiations. They have antibiotic resistance due to failure of antibiotics to penetrate the surface of biofilms and due to formation of resistant pheno-types (53).

Biofilms have always been described in association with chronic infections (66).

In tonsils,the irregular, crypt-like surface of the tonsil forms favourable surface for the bacterial attachment and biofilm growth (53).

Process of biofilm formation

Biofilm formation occurs in three different stages: attachment, maturation and dispersion.

The attachment step could be further categorized as a two-stage process: initial reversible attachment and irreversible attachment. The intial step of biofilm formation is attachment of bacteria to the surface. For this, bacteria should get close to a surface. There are several forces, both attractive and repulsive, that come into play for attachment of bacteria (67).The negative charges on the environment surface repels the negative charges on the bacterial surface. This repulsion is overcomed by van der Waals forces and also by the use of fimbriae and flagella on the bacterial surface that attach to environmental or host surface. This initial attachment mediated by type IV pili and flagella are important. Type IV pili-mediated twitching motilities enable the attached cells to aggregate and form microcolonies. The micrcolonies produces EPS.The production of EPS matrix signifies irreversible phase of bacterial attachment to a surface.

Once the first layer is established, cells of the same species or other species are recruited to the biofilm .Thus biofilm grows from a thin layer to multiple layers and form either ‘mushroom’ or ‘tower’ shape structure. Apart from recruiting bacteria from outside,the bacteria within biofilm also communicate with each other and take specialized functions. As the biofilm matures, more components like proteins, polysaccharides, DNA, etc. are secreted into the biofilm by the bacteria enclosed within the biofilm.

The last step that allows continuation of biofilm cycle and hence and important step for the bacteria is the dispersation. Biofilms are thought to disperse because of different factors like depletion of nutrients, heavy competition and overgrowth of bacterial population. The whole biofilm can get dispersed or just a part of it. New biofilms are formed at distant sites by the release of planktonic bacteria.

Detection of biofilms

Several bacterial species can develop a biofilm such as *Pseuomonas aerogenosa, Haemoplilus influenza ,Streptococcus pneumoniae,* and *Staphylococcus aureus* (6).Most of these organisms are responsible for otorhinolaryngological disorders. A number of tests are available to detect the biofilms. This is done by identifying the slime production. These methods include tissue culture plate (TCP) (68), Congo red agar (CRA) (69), tube method (TM) (70) ,bioluminescent assay(71) and light or fluorescence microscopic examination (72).

Tissue culture plate method (TCP)

The TCP assay (73) was described by Christensen *et al* (68) and is most widely used and is considered as standard test for detection of biofilm formation. The biofilm production in three different media is observed.

Isolates from fresh agar plates are inoculated into the media. It is incubated for 18 hours at 37^{o}C in standard condition .Isolates are then diluted 1in100 with fresh medium. Individual sterile wells of polystyrene, 96 well-flat bottom tissue culture plates wells are filled with 0.2 ml of the diluted cultures.

After incubation, content of each well are gently removed by tapping the plates. The wells are washed four times with phosphate buffer saline (PBS) to remove free-floating 'planktonic' bacteria. The sessile, tightly adherent organisms in biofilms are fixed with sodium acetate (2%) and then stained with crystal violet(o.1%). Excess stain is rinsed away by thoroughly washing with deionized water. The plates are then kept for drying. Optical density (OD) of stained bacteria is determined with a micro ELISA auto reader at wavelength of 570 nm. These optic density values are considered as an index of bacteria adhering to surface and in turn the biofilms.

Tube method (TM)

In this method, loopful of microorganism are taken from overnight culture plates and incubated for 24 hours at 37°C in 10ml of TSBglu media in test tube. The tubes are decanted and washed with PBS .The tubes are then dried and dried tubes are stained with crystal violet (0.1%). Tubes are thoroughly washed with deionized water to remove excess stain .Tubes are then dried in inverted position and observed for biofilm formation.

A visible film that line the wall and bottom of the tube is considered positive for biofilm formation. Ring formation at the liquid interface is not considered positive for biofilm formation. Tubes are examined and the amount of biofilm formation is scored as absent-0, weak-1, moderate-2 or strong-3. Experiments are performed in triplicate and repeated three times.

Congo red Agar method (CRA)

Freeman *et al* (69) had described an alternative method of screening biofilm .This

requires the use of a specially prepared solid medium with Congo red and 5% sucrose .

Black colonies with a dry crystalline consistency are considered positive result for biofilm formation. Weak slime producers usually remained pink. But occasional darkening at the centers of colonies is observed with pink staining around it. Again, colonies that are darkened but lack the dry crystalline colonial morphology indicate an indeterminate result. This experiment is also triplicated and repeated three times.

Much of the early works on biofilms were using scanning electron microscope. This technique utilizes solvents like alchohol or acetone to gradually dehydrate the specimen. The presence of water content in specimen is not compatible with the vacuum used with the electron beam in scanning electron microscope.

With the development of the confocal laser scanning microscope (CLSM) in 1980s, the investigator can examine biofilms in situ. Hence, the biofilm matrix is visualised unaltered and intact (74).For examining biofilms under CLSM, the organisms in the biofilms has to be stained with fluorescent stains.

Summary

Tonsillitis is one of the common infection affecting children especially under the age of 10 years. It is usually caused by GABHS, for which effective antibiotics are available, although it is known to cause serious complications such as acute glomerulonephritis or rheumatic fever. Recurrent tonsillitis in children is caused by the persistence of bacteria in the oropharynx especially the tonsils, which can be indication for tonsillectomy. There is dynamic equilibrium between commensal and pathogenic microorganisms in oropharynx with an inverse relationship between the amounts of two groups of organisms. It is assumed that asymptomatic children with no recurrent throat infection have more commensal flora when compared to children with recurrent tonsillitis. The better understanding of microbiological profile will help us in better management of throat infections. It can also contribute to recent trends in treatment of throat infection like bacterial replacement therapy with probiotics. There was no clear data available on the microbiological flora in two age-matched groupsof children with recurrent tonsillitis and asymptomatic group.

So the study was planned to describe the microbiological flora of the oropharynx in symptomatic and asymptomatic group. Furthur on ,to quantify the commensals in both groups and to assess the biofilm forming capacity in pathogenic group. This is a pilot study and information from the study will provide a broad idea about the relevance of the dynamic equilibrium of oropharyngeal microflorain both groups which woul be useful to design study on a larger population.

MATERIALS AND METHODS

This prospective observational study was conducted at Department of Otorhinolaryngology, Christian Medical College, Vellore . Approval of the Institutional Review Board at Christian Medical College was obtained in IRB Min no:9615(OBSERVE) dated 01.09.2015. Patients with recurrent tonsillitis were taken as study group and patients with other ear/nose complaints without throat symptoms presenting to the outpatient department were briefed about the research project and requested to participate in the study.

Study design

Observational prospective study.

Study period

September 2015 to August 2016.

Setting

The study was conducted at the Department of ENT ,and Department of Microbiolgy in Christian Medical College and Hospital ,Vellore. CMC Vellore is a tertiary referral centre in Tamil Nadu catering to patients from India and the neighboring countries. CMC has an average of 7500 outpatients and 2300 inpatients with daily bed occupancy of around 79% as per 2016 CMC records.

Participants

Consecutive children who present to the ENT unit -2 outpatient department with recurrent tonsils posted for tonsillectomy were included in study group after consenting. Age matched children without any complaints of recurrent throat infection who present to ENT OPD were taken as control group and both groups were evaluated as per clinical protocol.

Eligibility criteria

Inclusion Criteria –

Study group

Children < 14years with recurrent tonsillitis

- 7 episodes in 1 year/ 5 episodes in 2 years/ 3 episodes in 3 years.
- Not received any antibiotic treatment for a period of 4 weeks before coming to OPD

Control group

- Children <14 years with no complaints of throat and no evidence of recurrent throat infection

Exclusion criteria –

Study group

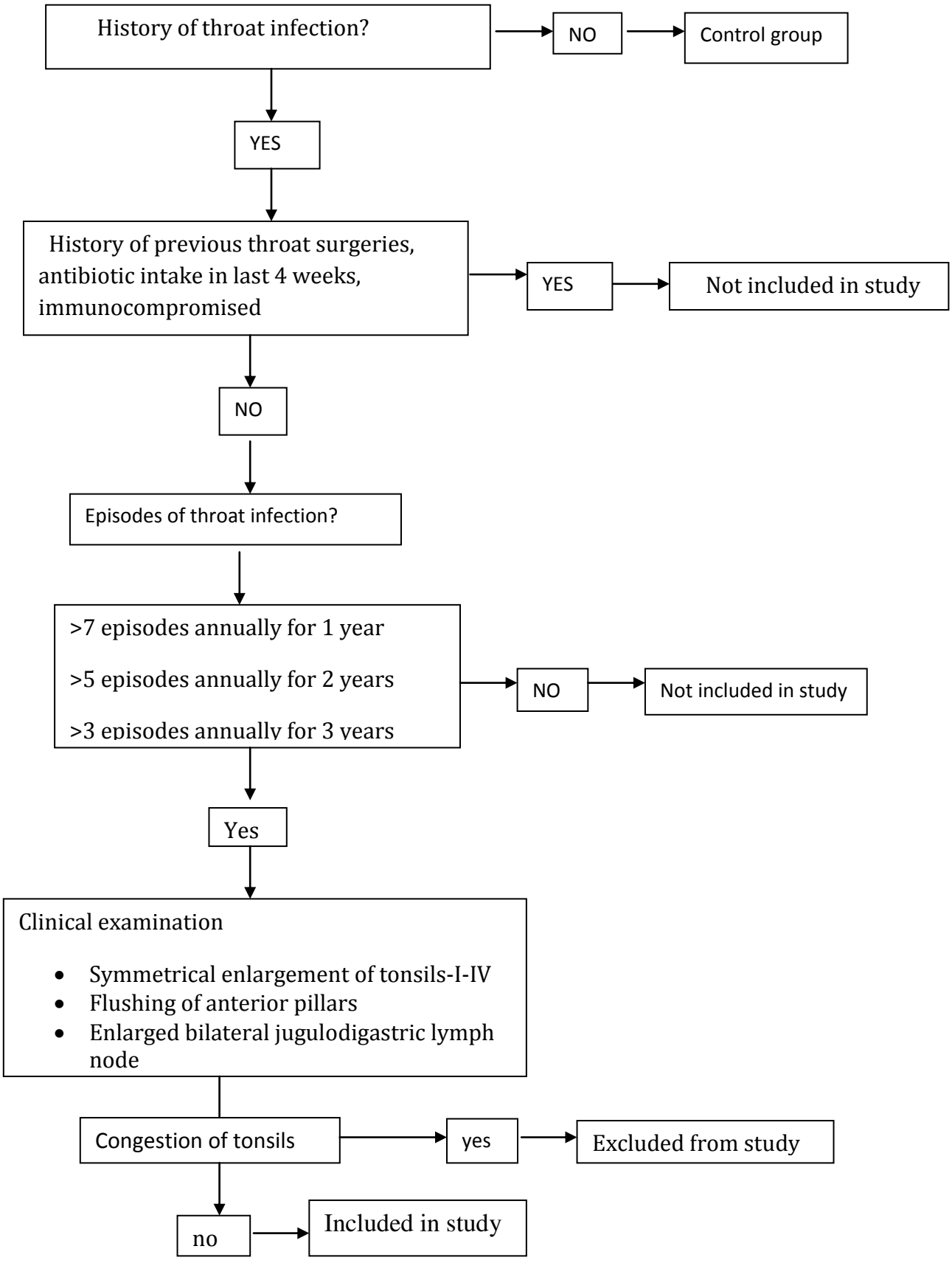
- History of upper airway surgery including tonsillectomy and adenoidectomy.
- Immuno-compromised
- Asymmetrical tonsils
- Congested tonsils
- Benign or malignant tonsillar tumour.
- Craniofacial anomalies.

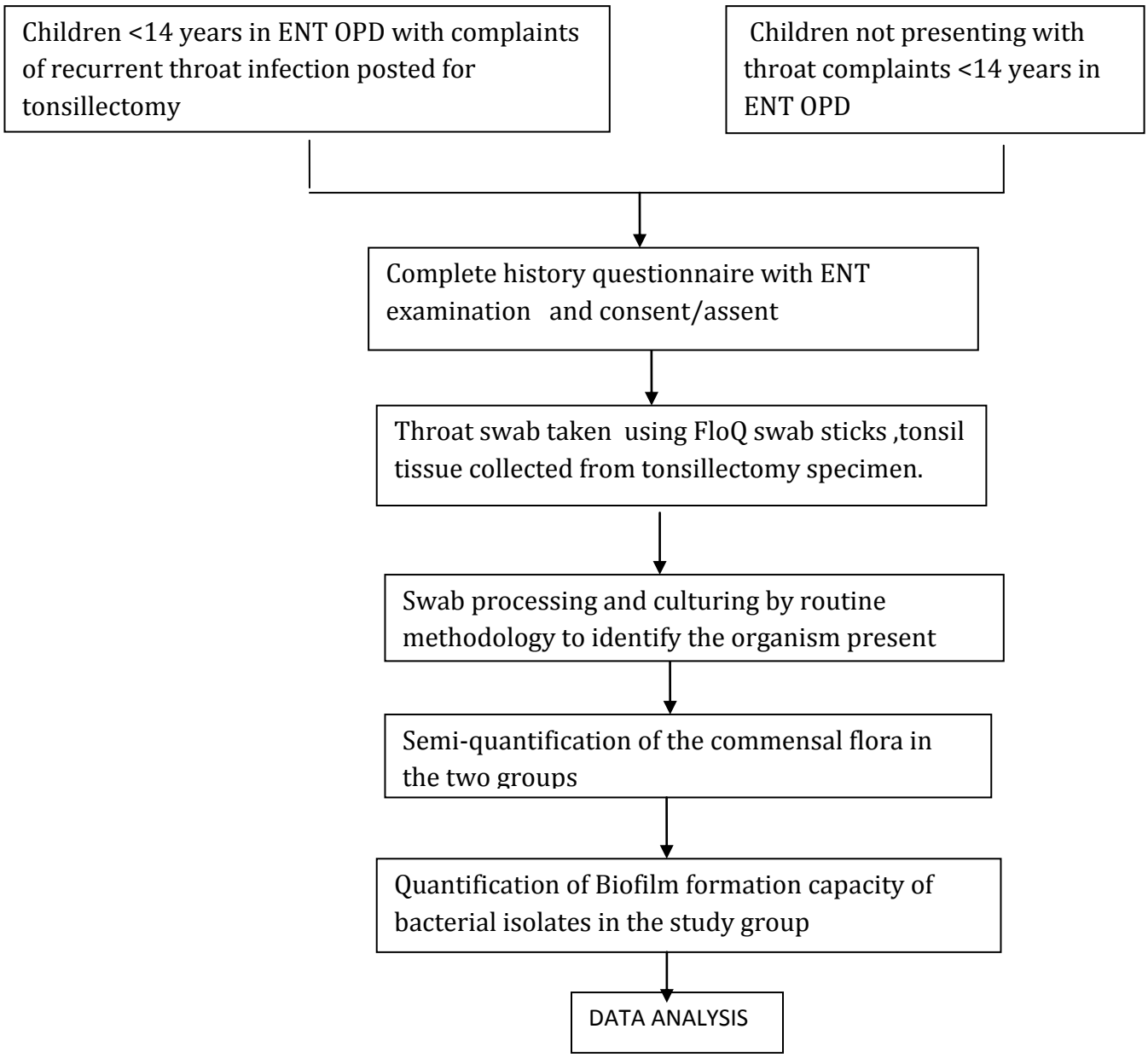
Control group

- History of antibiotic therapy within 4 weeks
- History of throat surgery
- Tonsillar enlargement
- Congestion of anterior pillar

Methodology

Children who satisfied the inclusion and exclusion criteria were invited to participate in the study and information sheet was provided. Those willing to participate, after consent and ascent, underwent a detailed history and ENT examination


History of throat infection?
NO
Control group
YES
History of previous throat surgeries, antibiotic intake in last 4 weeks, immunocompromised
YES
Not included in study
NO
Episodes of throat infection?
>7 episodes annually for 1 year
>5 episodes annually for 2 years
>3 episodes annually for 3 years
NO
Not included in study
Yes
Clinical examination
Symmetrical enlargement of tonsils-I-IV
Flushing of anterior pillars
Enlarged bilateral jugulodigastric lymph node
Congestion of tonsils
yes
Excluded from study
no
Included in study


Children <14 years in ENT OPD with complaints of recurrent throat infection posted for tonsillectomy
Children not presenting with throat complaints <14 years in ENT OPD
Complete history questionnaire with ENT examination and consent/assent
Throat swab taken using FloQ swab sticks ,tonsil tissue collected from tonsillectomy specimen.
Swab processing and culturing by routine methodology to identify the organism present
Semi-quantification of the commensal flora in the two groups
Quantification of Biofilm formation capacity of bacterial isolates in the study group
DATA ANALYSIS

The children selected and consented (consent from parents and ascend from children above seven years) for the study in both the groups underwent detailed history and standard ENT examination according to the proforma enclosed in the annexure. The children with recurrent tonsillitis were asked about the duration of throat pain and number of episodes per year. They were also asked history of snoring, last intake of antibiotics, ear symptoms and nose complaints.

Body mass index (BMI) and the socioeconomic status (SES) were calculated for all participants and are described below**.**

Body Mass Index (BMI) is a person's weight in kilograms divided by the square of height in meters. For children and teens, BMI is age-specific and sex-specific and is often referred to as BMI-for-age.

After BMI is calculated for children and teens, it is expressed as a percentile which can be obtained from either a graph or a percentile calculator.

Socioeconomic status

One of the most common findings in epidemiology is that individuals lower in socioeconomic status (SES) have poorer health than individuals higher in SES. This

relationship holds true whether health is measured as the prevalence rate of illness, the severity of illness, or the likelihood of mortality, and it is true for most types of diseases, as well as for many risk factors for diseases (75).

Parental SES may impact both parents' own health and the health of their children (76). Socioeconomic status (SES) is often measured as a combination of education, income and occupation (77).

Mother's education	Occupation of mother	Family income per month
Illiterate-1	Unemployed-1	>31,507(12)
Primary school ceritificate-2	Unskilled worker-2	15,745-31,506(10)
Middle school certificate-3	Semiskilled worker-3	11,817-15,753 (6)
High school certificate-4	Skilled worker-4	7878-11,816 (4)
Post high school-5	Clerical shop owner,farmer-5	4727-7877(3)
Graduate or post graduate-6	Semi professional-6	1590-4726(2)
Professional or honours-7	Professional-10	≤ 1589 (1)

Upper	Upper middle-	Lower middle	Upper lower	Lower
26-29	16-25	11-15	5-10	<5

The children were examined in ENT OPD setting with head light and Otoscopic examination.

Tonsils were graded according to Brodsky Grading Scale

It comprises of the following 5 grades: grade 0 (tonsils within the tonsillar fossa), grade 1 (tonsils just outside of the tonsillar fossa and occupy ≤25% of the oropharyngeal width), grade 2 (tonsils occupy 26%-50% of the oropharyngeal width), grade 3 (tonsils occupy 51%-75% of the oropharyngeal width), and grade 4 (tonsils occupy >75% of the oropharyngeal width) (78).

Throat swab was taken using COPAN FLOQ Swabs. The child sitting in front of the examiner was asked to open the mouth and say 'Ah'. The tongue was depressed by Lacs metal tongue depressor and two FLOQ swabs were rubbed over both the tonsils 3 to 4 times separately and then sent for microbiological examination.

If patient had history of snoring X- ray nasopharynx or diagnostic nasal endoscopy was done.

Using X-ray neck lateral view,the adenoid hypertrophy was graded as follows, according to the percentage of airway in nasopharynx:

Grade 1 <25%, Grade II-25%-50%, Grade III- 50-75%,Grade IV->75%.

FLOQ Swabs

It consists of a solid moulded plastic applicator shaft with a tip which is of various size and shape. The tip is coated with short Nylon fibers .They are arranged in a

perpendicular manner. This perpendicular arrangement is the result of a process called flocking. The swab is placed in te electrostatic field and fibres are sprayed onto the tip. The produces a highly absorbent thin layer (79).

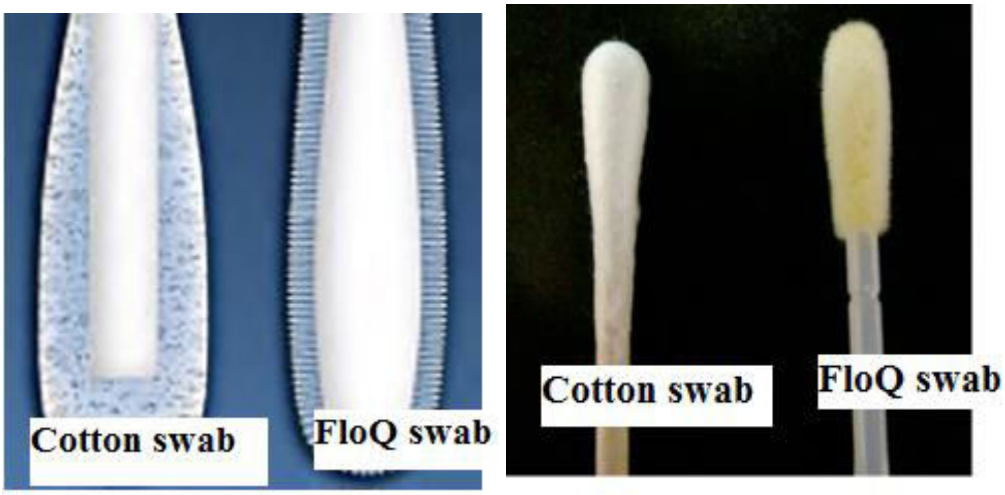


Figure 4:FloQ swabs

The cotton swabs are made of cotton fibers wrapped around the tip of a wooden stick. Because the cotton is highly absorbent, its dense inner core can trap cellular materials.

On the other hand, FloQ swabs are made of parallel short nylon strands that are flocked onto a plastic stick .Thus these swabs lack an inner core that can trap cellular materials.

Evaluation in microbiology lab

Two floQ swab one from each side of the tonsil were transported to the Microbiology lab within 2 hours after sample collection.

Isolation of aerobic bacteria

Samples were inoculation onto primary media-blood agar, chocolate agar once it reached the Microbiology lab. Plates were incubated for 24 to 48 hours and organisms were identified according to the standard operating protocols of the laboratory.

Individual microorganism were identified according to characteristic of colonies and confirmed using biochemical reactions per laboratory protocols.

Grouping of streptococci will be done using the co agglutination method after micronitrous acid extraction from the colonies which were suggestive of beta haemolytic streptococci. Haemophilus were identified using X and V factor requirement. Primary media used were

- Blood agar -for all Gram positive and negative bacteria.
- Chocolate agar - for isolation of Haemophilus species.

Isolates were stored at -70^0 C to test for biofilm formation in batches.

Antimicrobial susceptibility testing by Kirby Bauer disc diffusion method was done on pathogens known to cause tonsillitis according to and was interpreted according to CLSI guidelines.

Isolation of anaerobic bacteria

Samples were inoculated in primary anaerobic media as soon as samples reached the laboratory.

- Blood agar without base used for anaerobic culture (ANBA)
- Thioglycollate Broth (TB)

Figure 5: Anoxomat Mark II system

Figure 6: Three Gas cylinders attached to system

The anaerobic environment is provided by fully automated "Anoxomat" Mark II system. The plates are kept in an anaerobic jar and a cold catalyst (palladium catalyst) is added and tightly closed. By following the standard protocol for Anoxomat, anaerobic environment is created by evacuation and displacement method with hydrogen, nitrogen and carbon dioxide. The jars are then kept at 37°C for 5-7 days.

After 5-7 days, the plates were inspected for growth, if growth was seen on the primary anaerobic media, organisms were isolated and identified as per colony morphology and biochemical identification schemes as per standard protocols.

Semiquantification of bacterial isolates

Growth of each type of organism was graded as scanty, moderate or heavy depending on the number of colonies. (1-9 colonies- scanty, 10-99 colonies - moderate, >99- colonies-heavy)

Growing a Biofilm

To compare the biofilm formation of each organism, it is essential to find out the "doubling time" of that organism. Doubling time is defined as the time taken for an organism to double in number. It gives us a rough idea of how fast an organism can multiply in the ost environment,provided it gets all the nutrient required (ideal environment).

Doubling time of an organism can be found by plotting a growth curve. A loop full of the individual organism was taken and inoculated in MHB (Muller Hinton Broth) and incubated overnight at 37 ^{0}C. After 24 hours, 500ul of each overnight culture was re inoculated into 10 ml of fresh MHB and incubated at 37 degree C for few hours. Now the

OD (Optical density) of each organism was adjusted to 0.5 macfarland unit and diluted 20 fold in the MHB. This inoculums was used as the starting material for the growth curve analysis.

96 well U bottom plate was taken and 100ul of innoculum was pipette into each well in triplicate to reduce error. Plain MHB was used as blank control.

Now the plate is loaded into a Therom Scientific Multiskan GO Spectrophotometer. The reading was taken after every 10 minutes automatically by the instrument. The instrument has an in built shaker and an incubater which provides an optimum condition for the organism to grow. The reading was taken after 5 hours and 30 seconds. The instrument does a blank subtraction and average for the triplicates and give the data in the form of graph and excel.

Each isolate was incubated as long as the doubling time, which was different for each organism.

Staining

Add 125ul of a 0.1% solution of crystal violet in water to each well of the micro toiter plate and place the peg over it. Incubate it at room temperature for 10 to 15 minutes. Rinse the peg plate 3-4 times with water by submerging in a tub of water. Shake and blot vigorously on a stack of paper towels to get rid of

the excess cells and dye. Turn the peg plate upside down and dry for a few hours or overnight.Now add 125 μL of 30% acetic acid in water to each well of the micro titer plate and place the peg plate over it for 10- 15 minutes to solubilize the crystal violet. Remove the peg plate and place the microtitre plate which have the solubilized crystal violet in Spectrophotometer (Scientific Multiskan GO) and take the reading at 595 nm. After taking the reading the blank was substracted and the average of the triplicates were done by the instrument.

The biofilm formation, BF was calculated using the formula :

BF = AB-CW, where AB represents the optical density at 595 nm (OD 595nm) of a well containing stained attached bacteria and CW, the OD 595nm of the stained control wells containing bacteria-free medium. The samples were classified into 4 different group :

1) Strong - 0.300

2) Moderate – 0.22-0.299

3) Weak – 0.100-0.199

4) Negative - 0.100

Sample size calculation

The primary objective of this study is to compare the microbiological profile of children with recurrent tonsillitis with that of asymptomatic children. From our literature review, we could not identify studies characterizing the microbiological profile in pediatric population. Hence, we assume a total sample size of 80 (40 in control group and 40 in study group).

Statistical analysis

All study variables will be summarized using descriptive statistical methods (means, standard deviations, frequencies and percentages). Continuous outcome variables will be compared between the study groups (cases / controls) using independent two sample t-test. Chi-square test will be used for categorical variables. Absolute differences in the means and proportions of the outcome variables will be reported along with 95% confidence intervals (CIs).

RESULTS

There were a total of 80 patients, 40 patients with recurrent tonsillitis (study arm) and 40 asymptomatic patients (control arm) (Study:control = 1:1)

Results were analysed according to 3 groups

I Demographic profile

II Clinical profile

III Microbiological profile

A. Comparison of microbiota of both groups

B. Semi-quantification of commensals in both groups

C. Biofilm forming capacity in study group

I Demographic profile

I. 1 Age

All children less than 14 years were included in the study. Most common age group in both study and control group was less than 10 years. Both study group and control group were age matched and average age of children in study group was 7.12 and in control group was 8.17.

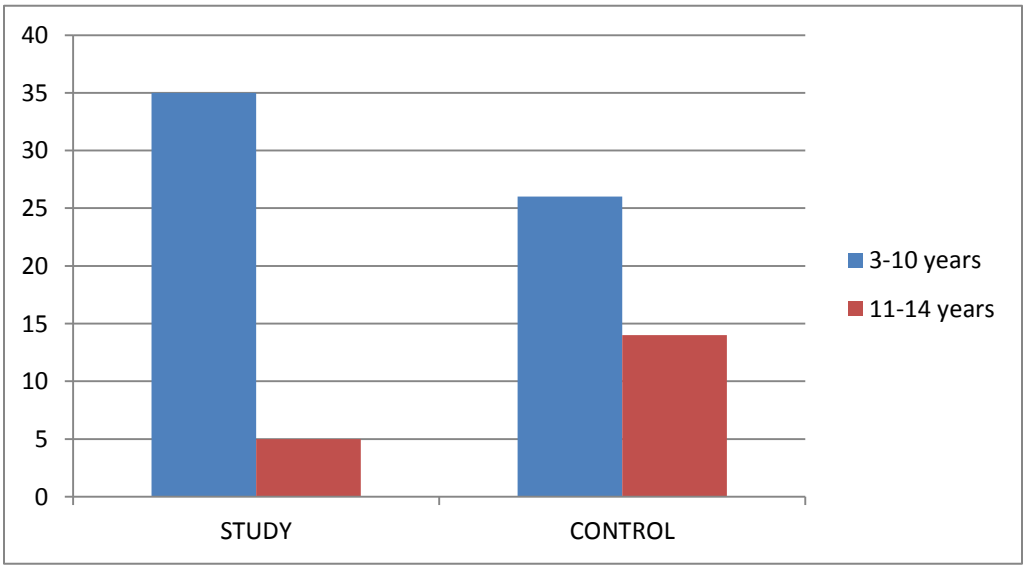


Figure 1: Age distribution in study group and control group with number of children in y-axis

I.2 Sex distribution

In our pilot study, most of the children were boys in both study and control groups.

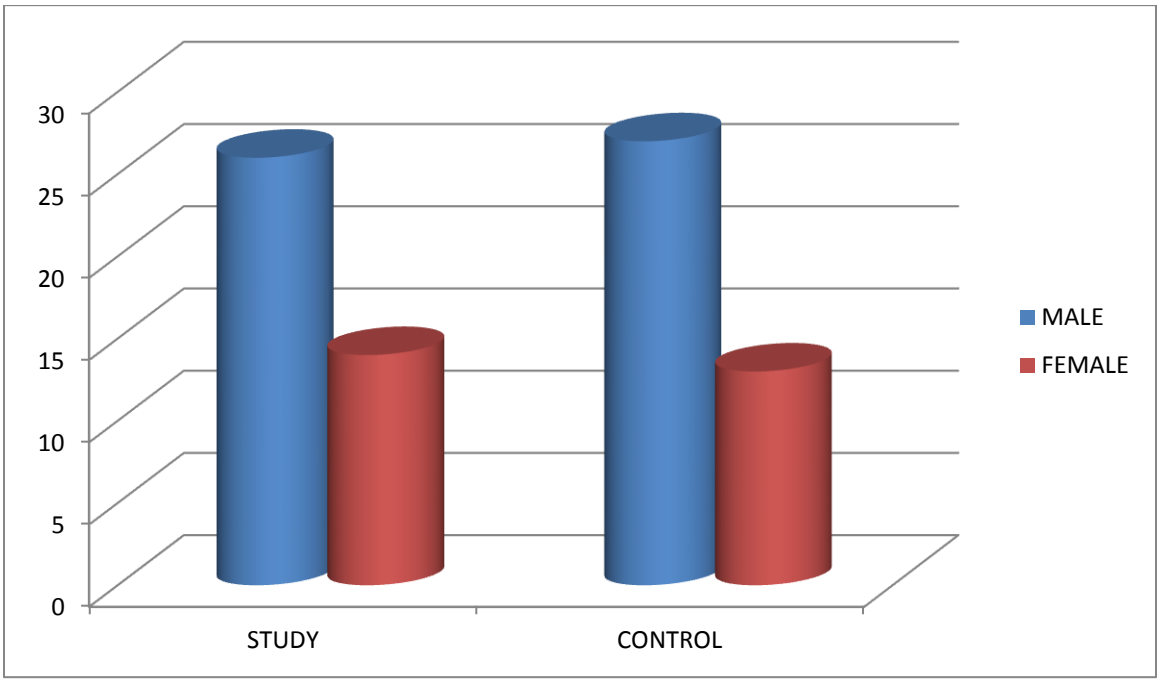


Figure 2: Sex distribution in study and control group with number of children in y-axis.

I.3 Geographic distribution

Most of the patients were recruited from Tamil Nadu.

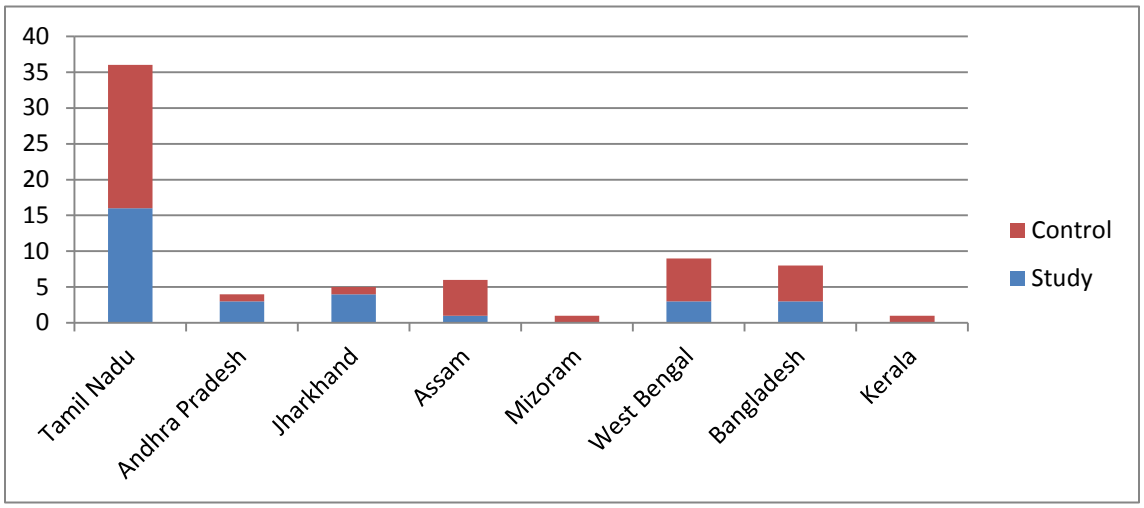


Figure 3: Geographic distribution of children in the study with number of children in y-axis

Patients from various states come for treatment in Christian medical college, but majority children were from Tamil Nadu (45%).

II .Clinical profile

II. 1 Socio-economic status (SES)

In this study, maternal occupation, education and family income were considered to assess the SES.Mother's education is scored from 1to 7, mother's occupation from 1 to 10, family income is taken 1-12. The total score is taken and SES is estimated:

Upper- 26-29, Upper middle 16-25,Lower middle 11-15, Upper lower 5-10, Lower <5

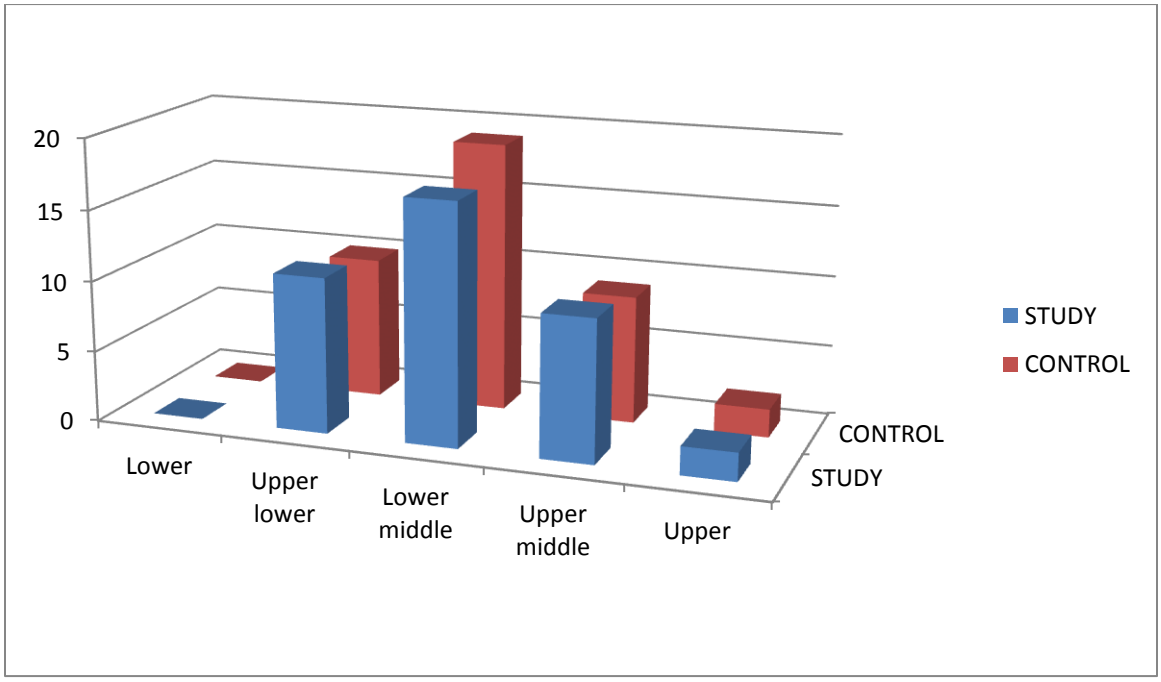


Figure 4: SES status distribution in study and control group with number of children in y-axis

In our study most of the children were from lower middle class (45%) .

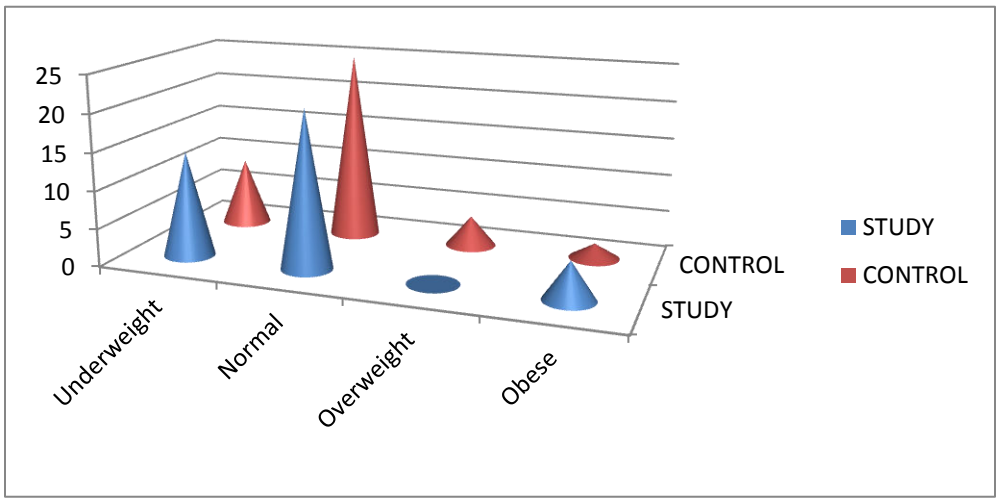
II.2 Body- mass index

In children, a high amount of body fat can lead to weight-related diseases and other health issues and being underweight can also put the child at risk for health issues.

After BMI is calculated for children and teens, it is expressed as a percentile which can be obtained from either a graph or a percentile calculator.

Weight Status Category	**Percentile Range**
Underweight	Less than the 5th percentile
Normal or Healthy Weight	5th percentile to less than the 85th percentile
Overweight	85th to less than the 95th percentile
Obese	Equal to or greater than the 95th percentile

Figure 5: Body mass index (BMI) in study and control group with number of children in y-axis



Though the BMI of most patients in study group and control group were normal, there were more underweight children in study group (35%) , though it was not statistically significant.

II. 3 Vaccination

All children in both groups were immunized for the age. We have looked at vaccination status according to age for BCG,OPV,DPT,MMR,HiB and Measles.

II.4 Duration of throat pain

According to Paradise criteria, duration of throat pain was classified as >7 episodes/ 1 year, > 5 episodes/ 2 years and >3 episodes/ 3 years.

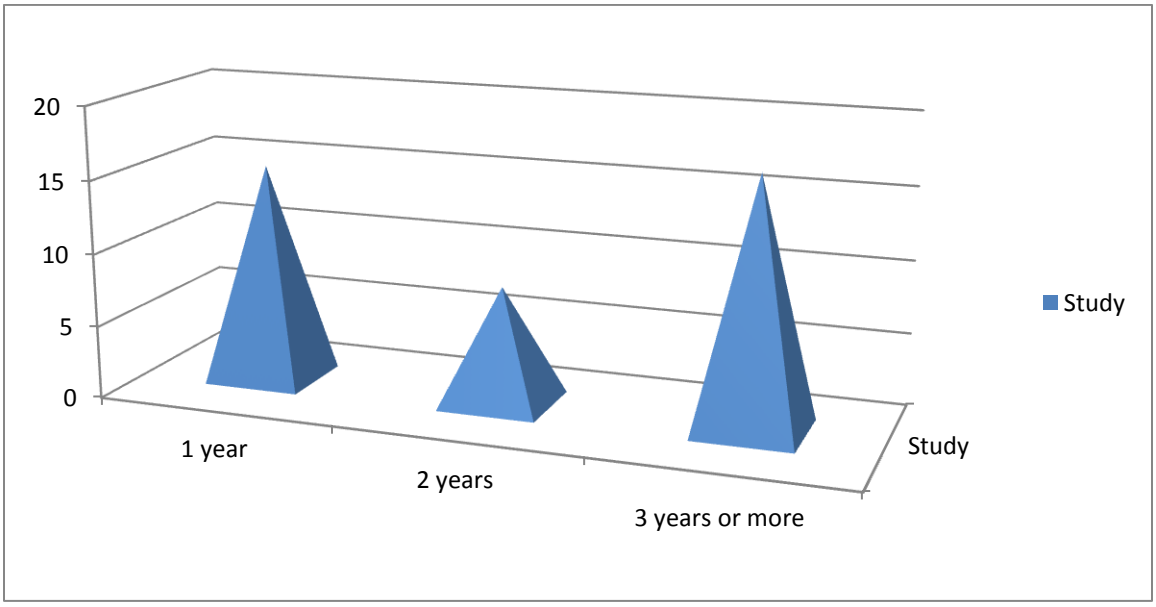


Figure 6: Duration of throat pain in children with recurrent tonsillitis with number of children along y-axis

In the study group,37.5% of children had only 1 year duration of throat pain.

II.5 Snoring

Snoring was a common complaint along with recurrent tonsillitis.

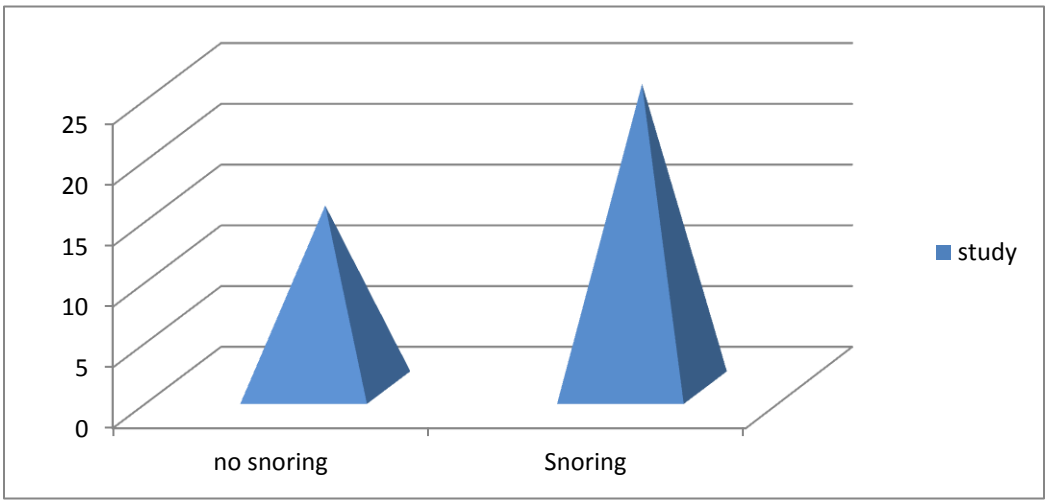


Figure 7: History of snoring in children with recurrent tonsillitis with number of children along y-axis

The usual cause of snoring is adenoid hypertrophy. Hence the adenoid hypertrophy was evaluated clinically in the study group.

II.6 Adenoid hypertrophy in children with recurrent tonsillitis

The adenoid hypertrophy grading according to x-ray nasopharynx lateral view

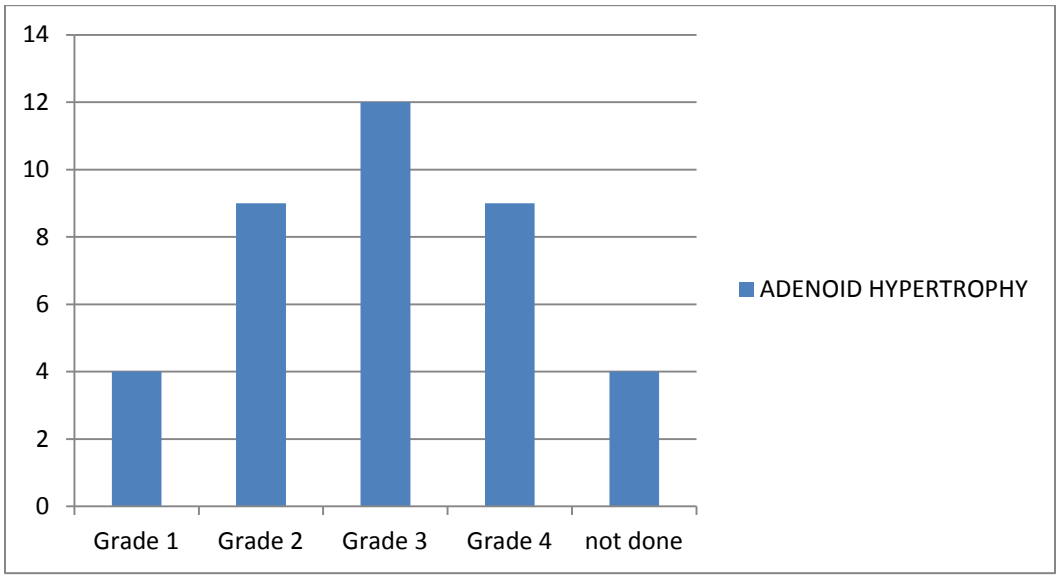


Figure 8: Adenoid hypertrophy in children with recurrent tonsillitis with number of children along y-axis

Majority (37.5%) of evaluated children had Grade 3 adenoid hypertrophy.

II.7 Ear symptoms

Few children had associated ear symptoms either ear block or ear discharge. Most common complaint among these children was ear block (83.3%).

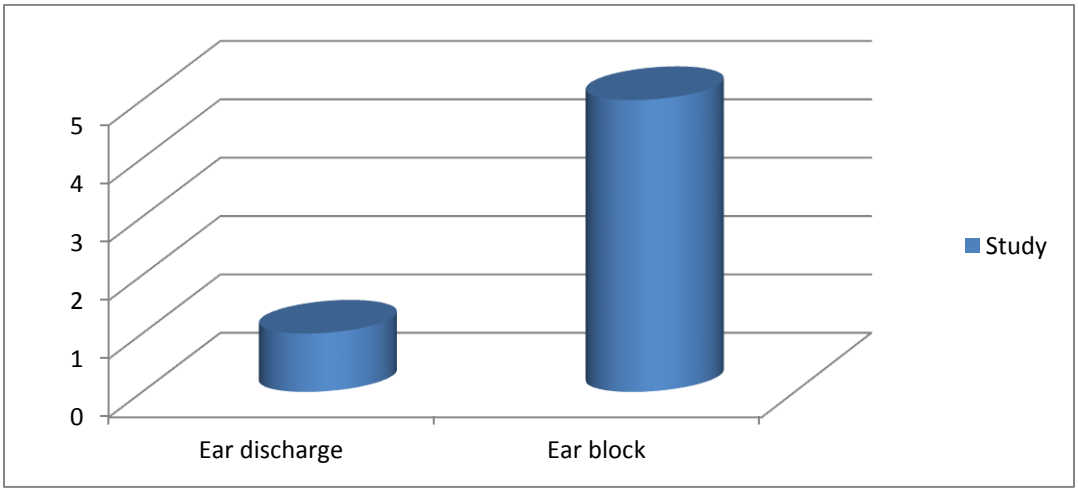


Figure 9: Ear symptoms in children with recurrent tonsillitis with number of children along y-axis.

II.8 Tonsillar hypertrophy in recurrent tonsillitis

Tonsillar enlargement was graded according to Brodinsky's grading system.

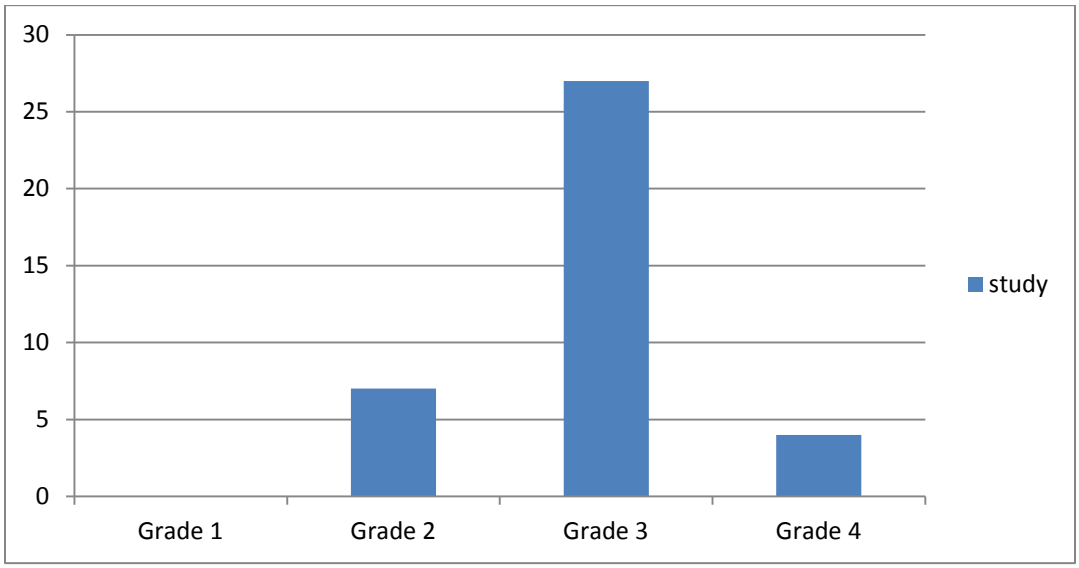


Figure 10: Grades of tonsillar hypertrophy in children with recurrent tonsillitis with number of children in y-axis.

Majority (67.5%) of the chidren with recurrent tonsillitis had Grade 3 tonsillar hypertrophy.

III. Microbiological profile

III.1. Microbiological profile in children with recurrent tonsillitis versus asymptomatic children

The microbiological profile of throat swab taken for both symptomatic and asymptomatic children and is as follows:

III.1.a Distribution of microorganisms in children with recurrent tonsillitis

From the throat swab, the micro-organisms cultured were classified as commensal and pathogenic bacteria in the symptomatic group.

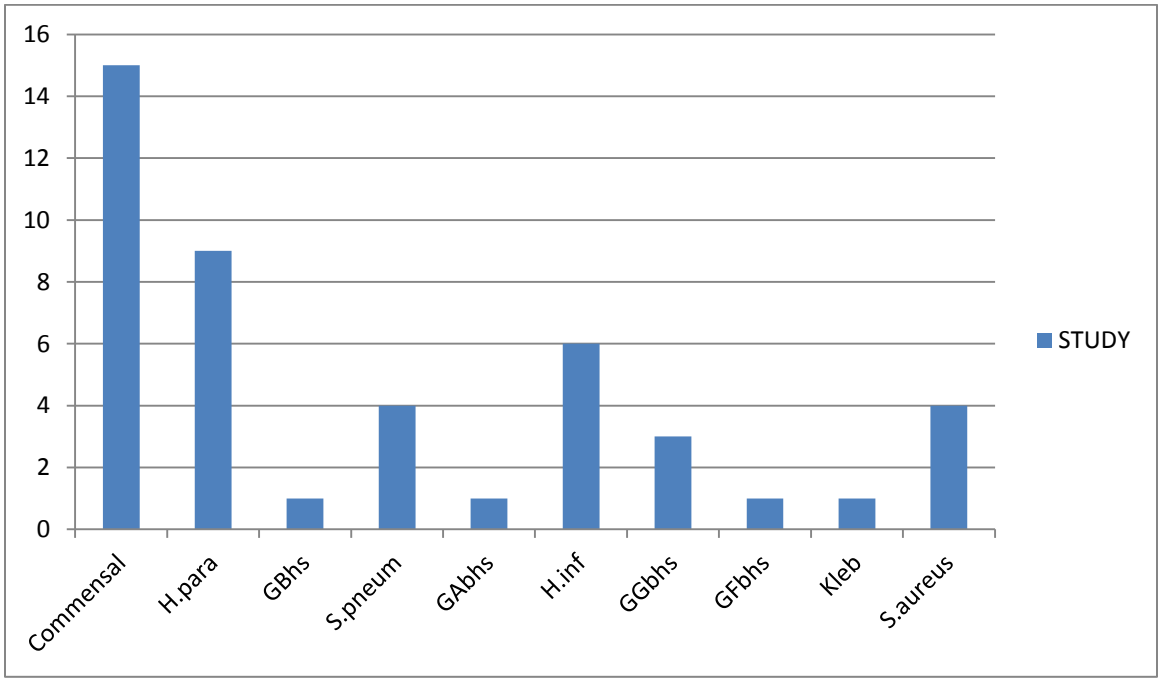


Figure 11:Distribution of micro-organisms in study group with number of children along y-axis

H.para- *Hemophilus parainfluenza*, Gbhs- *Group B hemolytic streptococci*, S.pneum- *Streptococcus pneumonia*, Gabhs- *Group A Beta hemolytic streptococci*, H.inf- *Hemophilus influenza,* Ggbhs- *Group G Beta hemolytic streptococci*, Gfbhs- *Group F Beta hemolytic streptococci*, Kleb- *Klebsiella* , S.aureus- *Staphylococcus aureus*

Throat swab had polymicrobial growth and commensal bacteria was found in maximum number of children**.**

III.1.b Distribution of micro organisms in asymptomatic children

From the throat swab, the micro-organism were classified as commensal and potentially pathogenic bacteria in the asymptomatic group.

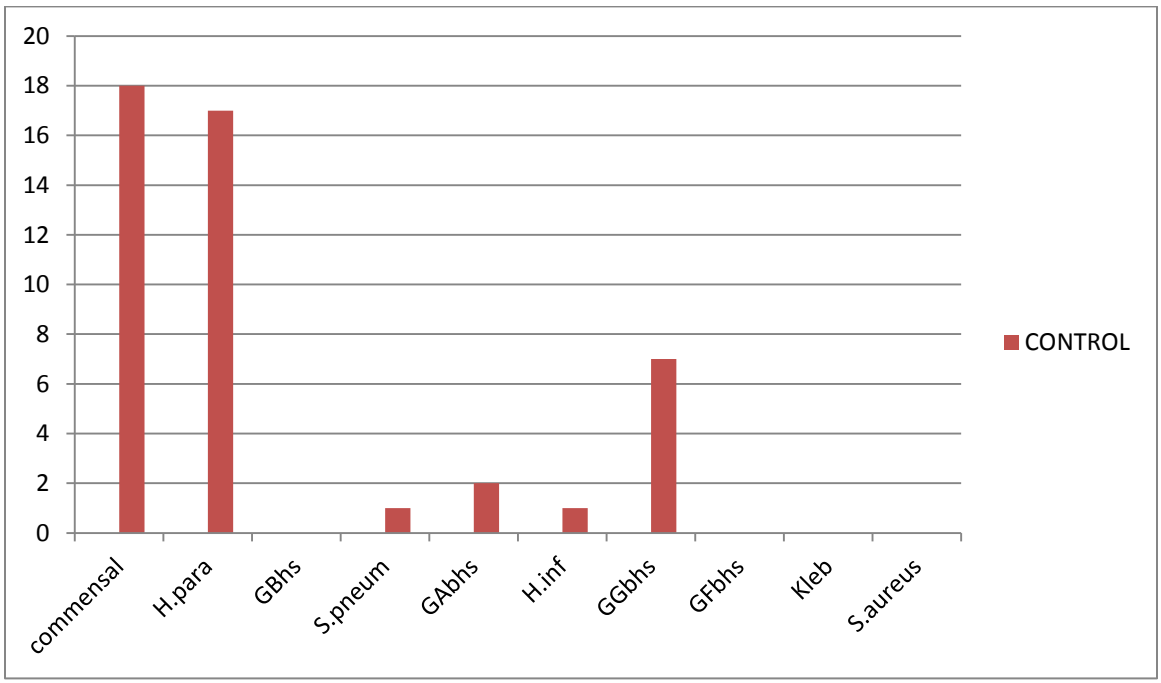


Figure 12: Distribution of micro organisms in control group with number of children along y-axis

H.para- *Hemophilus parainfluenza*, GBhs- *Group B hemolytic streptococci*, S.pneum- *Streptococcus pneumonia*, GAbhs- *Group A Beta hemolytic streptococci*, H.inf- *Hemophilus influenza*, GGbhs- *Group G Beta hemolytic streptococci*, GFbhs- *Group F Beta hemolytic streptococci,* Kleb- *Klebsiella* , S.aureus- *Staphylococcus aureus*

When microbiological profile was compared between the symptomatic and asymptomatic group, it was observed that potentially pathogenic bacteria was less in the control group.

However, when then microbiological profile of both study and control group were compared, there was no stastical significance .

III.1.c Age dependent trend in micro-organisms in study group

According to throat culture report of study group, they were grouped as normal flora and pathogenic organism according to the age.

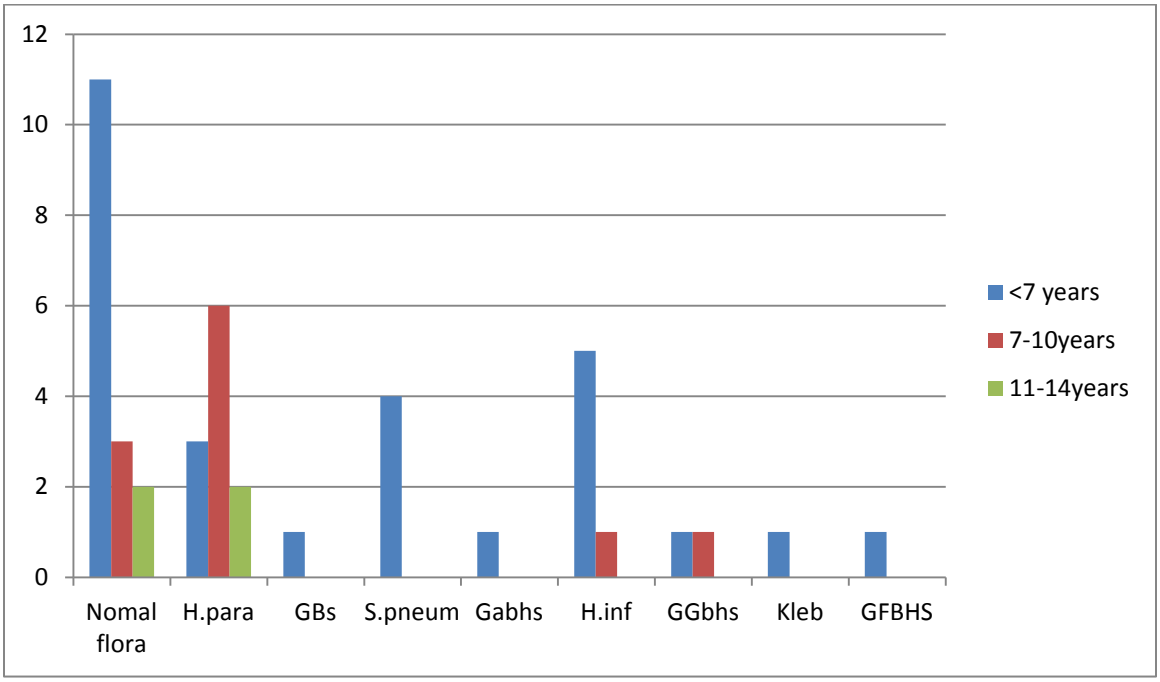
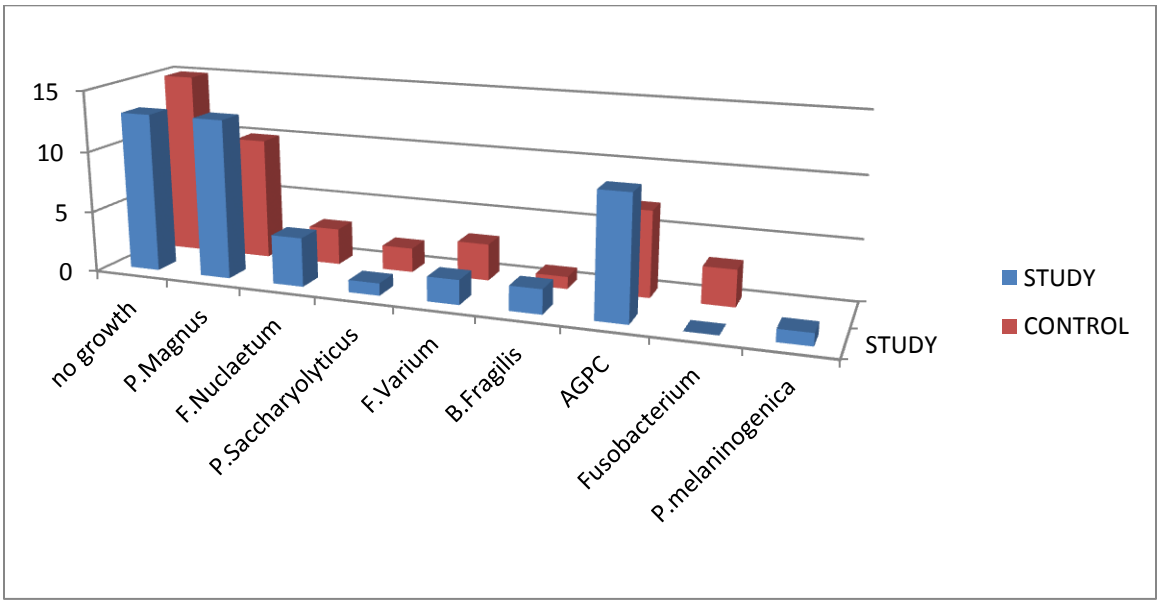


Figure 13: Age dependent trend of micro-organisms in children with recurrent tonsillitis with number of children along y-axis

There is polymicrobial growth in age < 7 years. As age increases, number of organisms in oropharynx decreases.

III.1.d Comparison of Anaerobes in study group and control group.



P.magnus- Peptostreptococcus magnus, F. nucleatum- Fusobacterium nucleatum, Psaccharyolyticus- Peptostreptococcus saccharyolyticus, F.varium- Fusobacterium varium, Agpc- Anerobic gram positive cocci , P.Melaninogenica- Prevotella melaninogenica

Figure 14: Distribution of anaerobes in two groups with number of children in y-axis

There is no statistically significant difference in the anaerobic organisms in both groups.

III.B Semi- quantification of commensals in both groups

Semi- quantification was done by observing growth of each type of organism and grading their growth as scanty, moderate or heavy depending on the number of colonies. (1-9 colonies- scanty, 10-99 colonies - moderate, >99- colonies- heavy).

III.B. a Semi-quantification of commensals in study group

In study group we looked at the commensals and semi-quantified their growth.

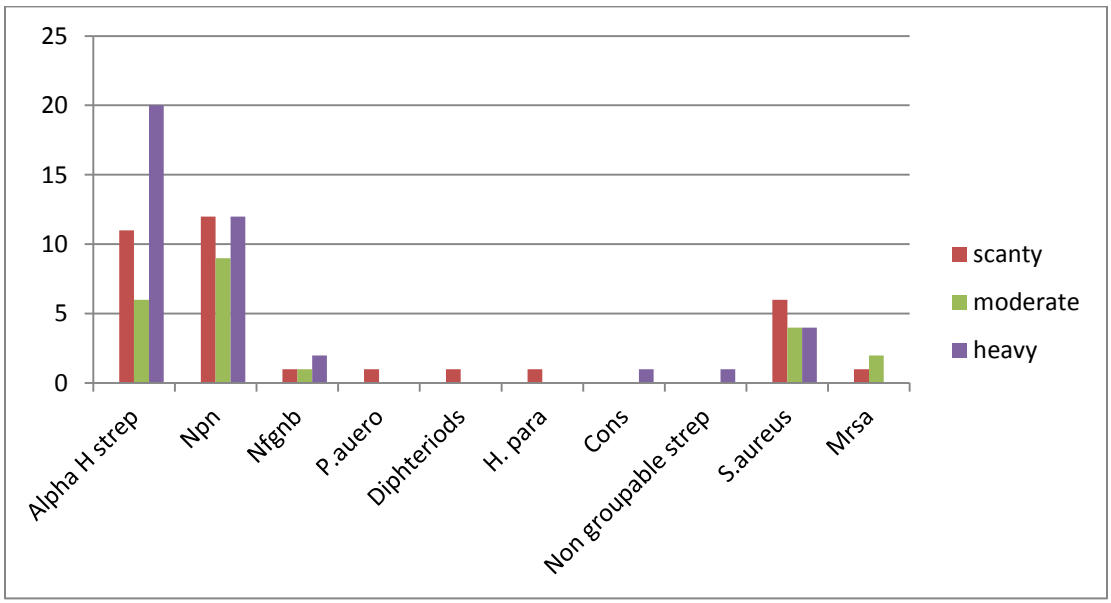
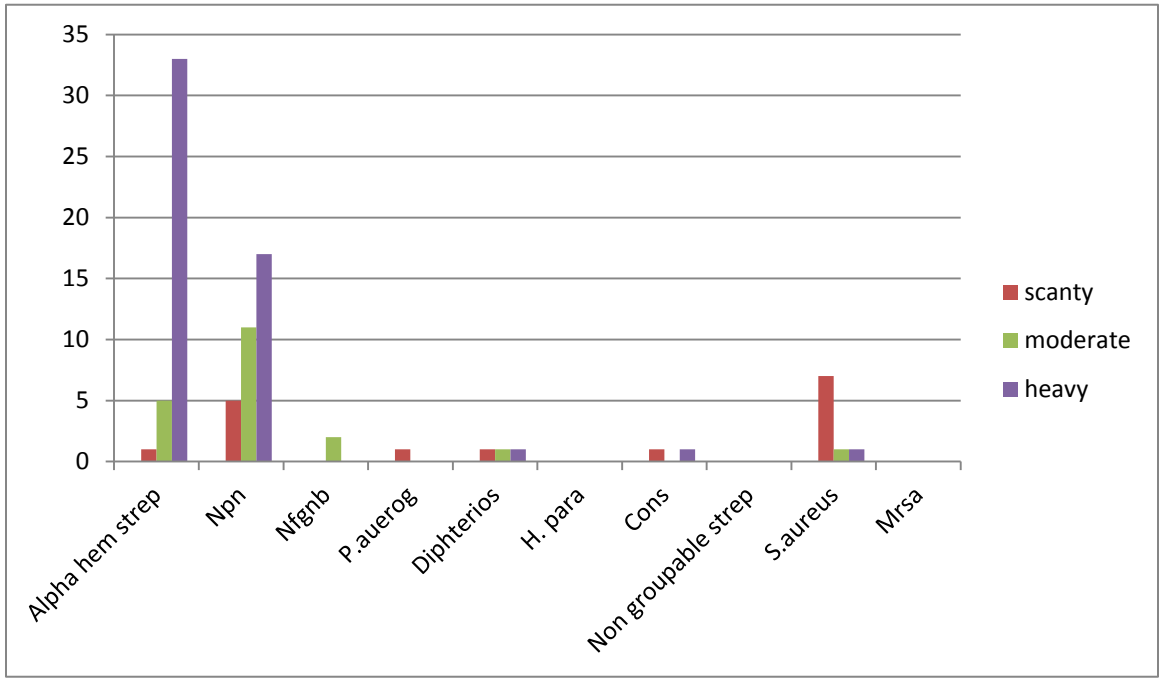


Figure 15: Semi-quantification of commensals in study group with number of children along y-axis

III.B. b Commensals in asymptomatic children



Alpha hem strep- Alpha hemolytic streptococci, Npn- Non pathogenic Neisseria, Nfgnb- Non fermenting Gram negative bacilli, P.aeuroginosa- Pseudomonas aeuroginosa, H.para- Hemophilus parainfluenza

Figure 16: Semi-quantification of normal flora in control group with number of children along y-axis

There is trend of heavy growth of commensal in asymptomatic group and it was more evident with *Alpha hemolytic streptococcus*, however there was statistically no significant difference between study and control group.

III.B. c Presence of alpha – hemolytic streptococci in children with recurrent tonsillitis and asymptomatic children

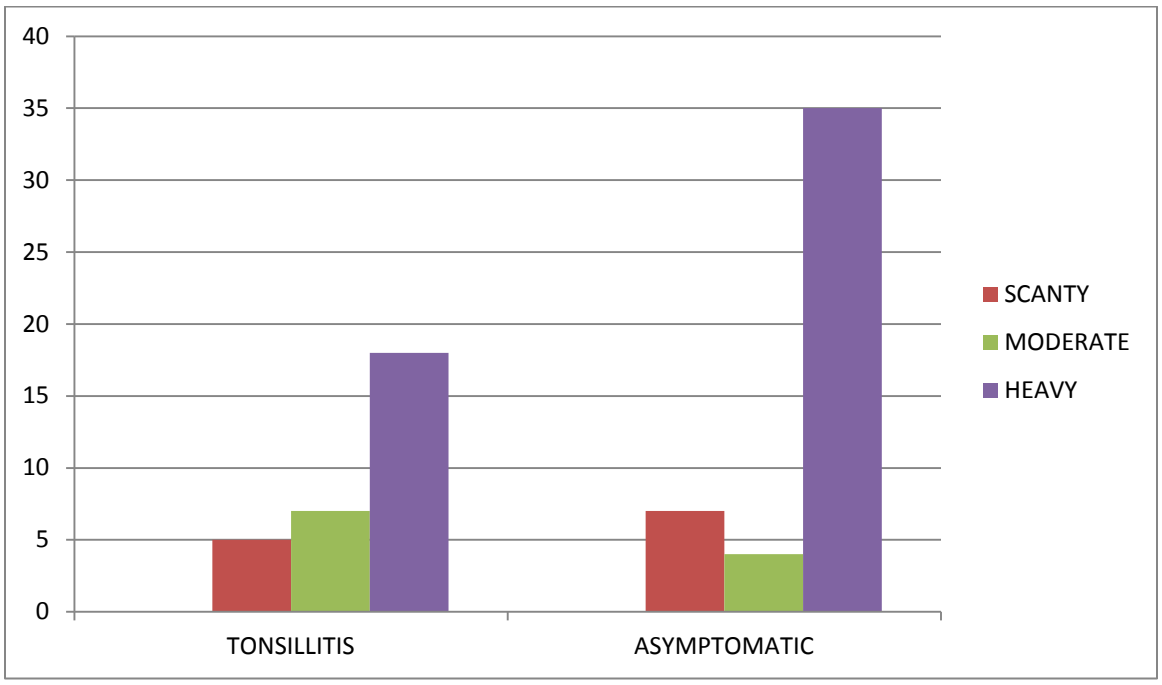


Figure: 17 Comparison of growth of Alpha Hemolytic Streptococci in both groups with number of children in y-axis

This was statistically analysed, and it was found that there was heavy growth of *Alpha hemolytic streptococci* in asymptomatic children when compared to study group (p- value- 0.024).

III.C Biofilm forming capacity of microrganisms in study group

Biofilm forming capacity of micro-organisms in study group were studied.Twenty isolates were retrived from the study group.

Table 1:isolates checked for biofilm forming capacity in symptomatic group.

Group B streptococcus- 1	Negative
Alpha- hemolytic streptococcus-7	Negative
Non-fermenting Gram negative bacilli-1	Negative
Group A beta hemolytic streptococcus-1	Negative
Pseudomonas aeuroginosa-1	Weak positive
Group F beta hemolytic streptoccus-1	Negative
Klebsiella Spp-1	Weak positive
Staphylococcus Aureus-2	1- moderate positive,1- strong positive
NH streptococcus-2	Negative
Non-pathogenic Neisseria-2	Negative
Streptococcus pneumonia-1	Negative

Biofilm forming capacity was found in three organisms in the study. These organisms were *Klebsiella spp* , *Staphylococcus auerus* and *Pseuomonas aeuroginosa* . Out of the three organisms, *S. aureus* had the strongest biofilm forming capacity.

Comparison of surface and core microbes in recurrent tonsillitis

Tonsillar core tissue taken from tonsillectomy specimen was cultured and compared with the surface pathogen obtained from throat swab. The samples were collected from 36 members of study group. The same pathogen was collected from swab and tonsil tissue in 24 samples for aerobes (66.67%) .

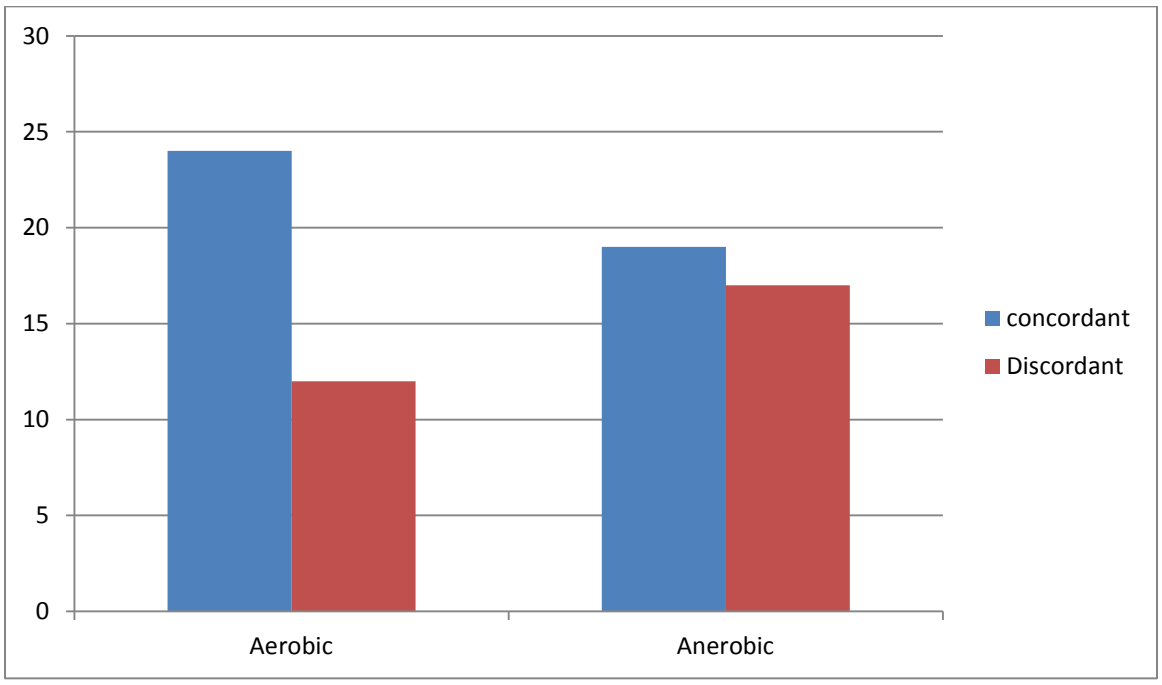


Figure 18: Comparison of core and surface pathogen in study group with number of children in y-axis

DISCUSSION

In this study, 40 children with history of recurrent tonsillitis were compared with asymptomatic children for their presentation and microbioloical profile.

In children, a dynamic equilibrium occurs between the commensals and potentially pathogenic organisms in the oropharynx where tonsils are situated. The balance between the two groups of bacteria can alter due to various reasons and can result in infection. And tonsillitis is one of the most common infections affecting children, especiallyless than 10 years. Therefore a better understanding of the microbiota of the oropharynx becomes relevant. In this study, 40 children with history of recurrent tonsillitis were compared with asymptomatic children for their presentation and microbioloical profile.

Majority of children with recurrent tonsillitis in this study (87.5%) were less than 10 years. The immunological activity of tonsils is greatest between 3- 10 years and later the tonsils starts involuting there by decreasing the episodes of tonsillar infection. Thus the study results are favoring this observation.

There were more boys in this study (65%) as compared to girls with tonsillitis. Most of the articles mention that there is no sex predilection. Both boys and girls have equal chance of getting recurrent tonsillitis.

Though most of the children were from Tamil Nadu, there were also children from north-east states and neighbouring countries of India like Bangladesh.

There is shown to be increased chance of infection in general, in underweight and malnourished children and children from low socio-economic status (80). This study had 35% children who were underweight, though numbers were more in the study group when compared to control group, it was not statistically significant.

Earlier, *Group A haemolytic streptococci* (GABHS) was considered the commonest causative organism of acute tonsillitis but now the trend is changing and other aerobic and anaerobic organisms are emerging (81). In a study by Bista et al , the more virulent organisms affecting tonsils were *S. pnuemoneae* and also *Beta haemolytic streptococci.* They also found that, the most common organism cultured from tonsillar tissue was

S. viridans. Investigators also observed that *Beta haemolytic streptococci*, a pathogen was not seen in the non infected group.

In this study, it was observed that potentially pathogenic bacteria were less in control group when compared to study group. The most common micro-organism in both groups was *Alpha hemolytic streptococci* and *Haemophilus parainfluenza*. The most common virulent organism found was *Streptococcus pneumonia* and was found more in study group. Other virulent organism is *Group A beta hemolytic streptococci*, though it is found in both groups, heavy growth is found in children with recurrent tonsillitis.In this study, heavy growth of *H.parainfluenza*was also found in both study and control group.
We also compared anaerobes between study and control group and there was no statistical difference between two groups. However, the most common anaerobe isolated from this study in both study and control group is Peptostreptococcus magnus.

The role of anaerobic bacteria in tonsillitis is hard to elucidate because these organisms are normally prevalent on the surface of the tonsils and pharynx as well as in the core of tonsils and adenoids, so that cultures taken directly, from these areas are difficult to interpret. Anaerobic bacteria are part of the normal on pharyngeal flora and are capable of interfering with the *in vitro* growth of other potential pathogens (82).

In a study by Brook et al(83), most common anaerobes isolated were Bacteroides spp , Gram-positive anaerobic cocci and 16 Veillonella spp.

In a study by Sternquist- Desatnik et al(84) , they compared the tonsillar tissue of patients undergoing tonsillectomy for recurrent tonsillitis(study group) and tonsillar hypertrophy for OSA(control group). There were 34 subjects, 17 in study group and 17 in control group between age group of 18-70 years. There was no significant difference between two groups considering aerobic and anerobic culture.

At birth, the oral cavity is sterile and colonization with a wide range of microbes occur within hours. And as age increase and with use of antibiotics, commensal will be replaced by pathogenic micro-organisms. In this study, polymicrobes were found in age group <7 years and as age increases number of micro-organims decreases.

Apart from finding micro-organisms we also made semi-quantitative estimates of cultural growth, since high counts are more likely to be related to pathogenicity than small numbers, which may merely represent healthy carriers.

There are very few studies semi-quantifying commensal in children with recurrent tonsillitis and asymptomatic children. In this study, there was trend of more commensal growth in asymptomatic group. But a clear finding was the heavy growth of alpha-

hemolytic streptococci in asymptomatic group when compared to the recurrent tonsillitis group. AHS are commensal inhabitants of the healthy nasopharynx that produce bacteriocins and have been investigated in clinical trials for their ability to inhibit pathogens through various mechanisms, including bacterial interference (54). Although some strains of AHS may cause infection , most strains are well tolerated and have been safely used as probiotic supplements in a number of clinical studies and are commercially available.

In this study, we also isolated *Methicillin resistant Staphylococcus aureus* (MRSA) in three children with recurrent tonsillitis. Though, MRSA is not a pathogen for tonsillitis. But the presence of MRSA in the tonsils acts as carrier and causes spread of the organisms to other individuals. They can also disseminate to other sites of body and cause infection there (85). Also, MRSA protect GABHS from penicillins by producing beta-lactamase.

The ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) are responsible for a substantial percentage of hospital acquired infections. They form a large majority of isolates that are resistant to antibiotics (86). The cause of antibiotic resistance are many including formation of biofilm (87).

Biofilm matrix provides a shield that protects the bacteria from the activity of the drugs (87).

In this study group ESKAPE isolates found were *S.aureus*,[scanty (32.35%), moderate(2.94%) and heavy (2.94%)] ,*P. aeuroginosa* [scanty(2.94%)] and *Klebsiella* [scanty (2.94%)]. Most of the patients were out-patients, hence theses organisms were found in less number.

In this study, 20 isolates were retrieved from the 13 patients in study group, with biofilm formation in four isolates (20%). These four isolates were recovered from three patients. Strongest biofilm forming capacity was shown by *S. aureus*. There was weak biofilm forming capacity by *P.aeuroginosa* and *Klebsiella* spp.

It has been demonstrated that several bacterial species are able to develop a biofilm, The most common micro-organism isolated from otorhinolaryngologic disorders include *Streptococcus pneumonia, H. influenzae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (88).

.

In a study by Mitchelmore et al(89), analysis of samples from individual patients revealed differences in the bacterial flora of the tonsil core and the tonsil surface. In this study, same isolates were found in both throat swab and tonsillar tissue in 60% in aerobes and 47.5% of anaerobes. Anaerobes are very sensitive organism and very difficult to retrieve from throat swab. The throat swab was transported to microbiology lab within 2 hours and we could identify same anaerobe from throat swab and core of tonsil in 19 samples (52.78%).

Conclusion

The micribiological profile of oropharynx in children with recurrent tonsillitis has its relevance due to factors like, bacterial interference and antibiotic resistance. This study was done to compare the microbiological profile of children with recurrent tonsillitis and asymptomatic children

In comparison, there was a trend of more heavy growth of commensal in the asymptomatic group as compared to symptomatic group, Though when analysed statistically, there was no significant difference in the profile of the microorganisms in two groups. Being a pilot study, it was concluded that a further study with larger sample size will be required to confirm the observation. It was interesting to note that, there was statistically significant increase the heavy growth of alpha- hemolytic streptococci, which is a commensal in the asymptomatic group as compared to symptomatic group. It was also observed that the amount of potentially pathogenic bacteria in the asymptomatic group less compared to tonsillitis group.

In summary this pilot study showed some interesting observations with regard to microbiota of the throat which is worth exploring using a study with larger sample size.

Limitations

This study had certain limitations. The tonsil surface is in contact with the bacterial flora of oral secretions.Thus the organisms isolated from the surface swabs might be the surface colonized bacteria rather than the actual pathogenic agents.

Some of the micro-organisms like Heamophilus spp are very liable and hence could not be retrieved during biofilm study.

Vaccination history could not be accurately obtained from parents of older children (>10 years).

One of our exclusion criteria was that the patient should not be on any antibiotics for atleast a period of 4 weeks. Since this a very common drug prescribed for even minor ailments, some of them might have actually taken this medication and were not aware of the same.

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ANNEXURE

INFORMATION SHEET

Christian Medical college, Vellore

Department of ENT

Study title: To compare good bacteria and bad bacteria in the throat of children with repeated throat infection to asymptomatic children

Thanking you for taking the time to read this Participant information sheet and consent form. We would like to invite you to participate in a research project that is explained below.

The good and bad bacteria which causes infection, colonises the throat in children. Both the bacteria are found in children but the type and the quantity will be different in children with repeated throat infection and normal children. The purpose of this study is to find out the good and bad bacteria in throat of children with repeated throat infection and to compare it with healthy children. The study also aims to find out the quantity of good and bad bacteria in the throat of both group of children. It will also determine if bad bacteria causes recurrent infection has any ability to form covering to protect themselves.

Procedure: Swabs will be taken from the children's throat by gently rubbing the swab sticks covered with cotton, on the tonsils. Taking a swab from the throat can cause gag, but no other discomfort. And it will be done by someone who is experienced in swab collection. This will be sent for analysis in the lab.

Benefit of the study: The results obtained from the study are expected to improve the knowledge about the bacteria that cause of recurrent throat infection in children. In future, this will help in improving the approach towards the management of such cases.

The information collected from your child will be kept confidential, analyzed separately and results published in standard medical books, without revealing your identity.

The child's participation in the study is voluntary and you are free to withdraw at any time without giving any reason. Refusal to participate in the research study will not involve any penalty or loss of benefits to which you are otherwise entitled.

DATE:

Dr.Chinnu Ann Stephen
Ph no: 8754144702

Informed Consent form to participate in the study

Study Title: To compare good and bad bacteria in the throat of children with recurrent throat infection and healthy children

Subject's Name: _____

Age/date of birth: _____

Hospital Number: _____

I _____ am willing to allow my Son/daughter _____ to take part in the study.

OR

I _____ Son/ Daughter of _____

is willing to take part in the study

(i) Declare that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions.

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

iii) I understand that the Sponsor of the clinical trial, others working on the Sponsor's behalf,the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any

further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published.

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

(v) I agree to take part in the above study.

Name/ Thumb impression:

Signature:

Date:

Name of witness/ Thumb impression:

Relation to participant:

Date:

Signature of the Investigator: _____

Date: _____/_____/_____

CLINICAL RESEARCH FORM/ PROFORMA

Serial no:

STUDY: COMPARISON OF MICROBIOTA IN CHILDREN WITH RECURRENT TONSILLITIS AND IN ASYMPTOMATIC CHILREN-PILOT STUDY

INVESTIGATOR-Dr. CHINNU ANN STEPHEN

NAME:

AGE:

GENDER: male (1) /Female(2)

HOSPITAL no:

DATE OF ENROLMENT:

ADDRESS:

CONTACT no: | | | | | | | | | | |

Occupation of mother: [ ]

1. Unemployed-1
2. Unskilled worker-2
3. Semiskilled worker-3
4. Skilled worker-4
5. Clerical, shop owner ,farmer-5
6. Semi professional-6
7. Professional-10

Mother's education: [ ]

1. Illiterate -1
2. Primary school certificate-2

3. Middle school certificate-3
4. High school certificate-4
5. Intermediate or post high school-5
6. Graduate or post graduate-6
7. Professional or honors-7

[1]

Family income per month (in Rs.) [ ]

1. >31,507 (12)
2. 15,745-31,506 (10)
3. 11,817-15,753 (6)
4. 7878-11,816 (4)
5. 4727-7877 (3)
6. 1590-4726 (2)
7. ≤1589 (1)

Socioeconomic class

Upper 26-29

Upper middle 16-25

Lower middle 11-15

Upper lower 5-10

Lower <5

Kuppuswamy Score [ ][ ]

HISTORY:

1) number of episodes of sore throat in 1 year. [ ]

number of episodes of sore throat in 2 years. [ ]

number of episodes of sore throat in 3 years. [ ]

YES	1	NO	0

a)High grade fever		
b)malaise		
C)difficulty in swallowing		

2) Each episode-any features-

3) absentees from school

if yes,how many days.

4) intake of any antibiotics, (YES/NO)

i) If yes,

How many times a year?	
How many years?	
Specify antibiotic, if possible	
Last intake of antibiotics(in weeks)	

5) age of first episode of sore throat

6) history of snoring?(YES/NO)

If yes,

Snoring heard throughout the floor/house	
Snoring heard in next room with door closed	
Snoring heard only in the bedroom	

7) history of any ear discharge?

8) history of ear blocking sensation?

9) crowded accommodation- no. of people in the house
10) siblings with similar complaint
11) poor nutrition.
12) sorethroat following URI.
13) last episode of throat infection
14) previous history of any throat surgery
15) history of any allergy?
16) history of any immunodeficiency?

IMMUNISATION:

1	BCG	
2	OPV	
3	DPT	
4	MMR	
5	HiB	
6	Measles	

TREATMENT/SURGICAL HISTORY:

FAMILY HISTORY: No. of siblings ☐

GENERAL EXAMINATION:

Height ☐☐☐cm Weight ☐☐ Kg

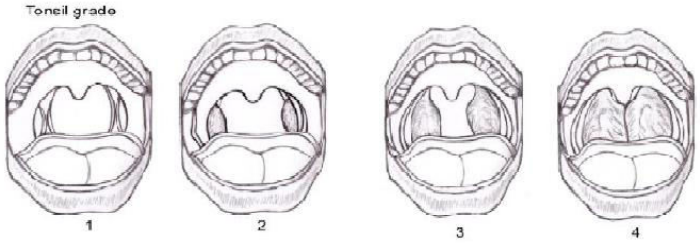
EXAMINATION:

FACE any craniofacial abnormalities.

ORAL CAVITY

Cleft palate	
Cleft lip	
Bifid uvula	

Grading of tonsils



< 25%		< 50%		< 75%		> 75%	
Grade 1	1	Grade 2	2	Grade 3	3	Grade 4	4

- asymmetry of tonsils (Y/N) ☐
- congestion of tonsils (Y/N) ☐
- Debris in tonsils(Y/N) ☐
- Anterior pillar congestion(Y/N) ☐

NECK - palpable jugulodigastric lymph node >2cm, (Y/N) ☐

if yes,

unilateral ☐ bilateral ☐

tender ☐ Non-tender ☐

NOSE -Septum- central ☐ Right ☐ Left ☐

Pale mucosa (Y/N) ☐
Nasal discharge (Y/N) ☐
PNS tenderness (Y/N) ☐

EARS- any discharge (Y/N)
Tympanic membrane perforation (Y/N)
If yes,

Right

Left

Dull looking tympanic membrane (Y/N)

GRADE 1	<25%	
GRADE 2	26-50%	
GRADE 3	51-75%	
GRADE 4	>76%	

INVESTIGATIONS :Adenoid x-ray, DATE

Culture growth:

Aerobic: Anerobic:

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Semi-quantification:

1	Scanty (1-9colonies)
2	Moderate (10-99colonies)
3	Heavy (>99 colonies)

Biofilm:Yes/No

1	Non proudcers	OD ≤ ODb
2	Weak producers	ODb<OD ≤ ODb
3	Moderate producers	2ODb<OD ≤ 4Ob
4	Heavy producers	4OD<OD

STANDARD OPERATING PROCEDURE FOR THROAT SPECIMENS

1. PURPOSE

To describe in detail the collection and transport of throat samples for microbiological examination.

THROAT

Specimen received as swab in sterile test tubes.

3.PROCESSING

All specimen are processed inside the biosafety hood

To describe in detail the Microscopic examination, culture and antibiotic susceptibility test of pathogens isolated from throat samples.

4.MICROSCOPIC EXAMINATION

a. Throat

- Smear examination for throat swab is done if diphtheria or Vincent's angina is suspected as the organisms have a distinct morphology.
- If there is too little material, make a note of this since such specimens are not really satisfactory. With one swab, prepare distinct smears; one for Gram stain, and if diphtheria is suspected,two more, for Albert's and Ponder's stains,which

may show the metachromatic granules characteristics of these bacilli more clearly.

- If Vincent's angina is suspected, counter stain with dilute carbol fuschin (Gram stain) to make the spirochetes more visible.
- Make a note of those organisms which predominate. In case of suspected diphtheria, send the report of the direct smear finding immediately whether positive or negative.
- If the smear has been unsatisfactory,request another specimen at once, suggesting that a bit of the membrane, if present, be teased off and sent.

5.CULTURE PROCEDURE

a.Throat

- Inoculate throat swab onto Blood agar (BA) plates only. Sheep blood is recommended for the isolation of *S.pyogenes* and S. *pneumonia* as colony size morphology and even minimal hemolysis is better appreciated on this.
- Inoculate Chocalate agar (CA) for specimen from immunocompromised patients, child health units and age of 10 or below from all units.
- If diphtheria is suspected or *C.diphteria* are seen in the irect smear, include a Loeffler's serum slope (LSS) and serum tellurite agar (STA). Inoculate smear

swab on to a second BA. LSS incubated at CO2 for 6 hours. After 6 hours subculture on to serum tellurite agar (STA) and blood agar (BA).

- Make smears and examine (Gram stain's, Albert's and Ponder's stains) from LSS after 4-6 hours and if positive or negative for *C.diptheria* ,subculture onto BA3 and STA2 and follow up for *C.diptheria.*
- If YLO are found in the direct smear, inoculate Sabouraud's DextroseAgar with antibiotics (SAB) incubate BA and CA in CO2 atmosphere. Include MA, and SAB for throat swab sent for survillence culture.
- For detection for Beta Hemolytic streptococcus, cut BA at the area of the first streaking, since more clear-cut hemolysis may be obtained from deeper colonises at the cut area due to the activity of both oxygen stable and oxygen labile hemolysis. It is important that sheep blood be used in BA for recognizing beta hemolysis streptococci.

5. CULTURE FOLLOW- UP

THROAT: POTENTIAL PATHOGENS INCLUDE:

- Beta- hemolytic streptococci (Groups A,C,G)
- C.diphteria
- Aranobacterium hemolyticum
- Borrelia vincenti/ fusobacterium fusiform (By smear only)
- Grade the growth of the organisms as scanty(sc), Moderate(mod) or Heavy(H). Proceed to identify the organisms according to protocols standardized in the laboratory.

- *Candida albicans* (especially from immunosuppressed patients, those from Haemology and whenever grown in moderate numbers in the absence of other pathogens or in heavy numbers with or without other pathogens)
- *Fo*r survellience cultures, follow up all organisms grown on the media and work up as potential pathogens except non-pathogenic Neisseriae and diphteriods.
- Haemophilus influenza, Haemophilus parainfluenzae, Haemophilus parahemolyticus (These are reported along with common- can be a colonizer)

7.REPORT

Throat

Smear:

- Gram stain are not done for throat swabs except on special request on when Diphteria or Vincent's angina is suspected.
- In case of suspected Diphteria, Gram stain, Alberts stain and Ponders stain is done and reported immediately as positive or negative.
- In case of suspected diphtheria, send the direct smear finding immediately, whether positive or negative.
- Smear is reported if Vincent's angina organisms are present.

Culture:

- Report on pathogens within 48 hours, giving the growth as heavy, moderate or scanty.
- These would include usually Beta- hemolytic streptococci (Group A,C,G)

- C.*diphteria, B.pertussis,N.meningitidis and A.hemolyticum.*
- If S.aureus,S.pneumoniae and H.influenzae are found in clear predominance, report them and indicate that these are not confirmed agents of pharyingitis reported as colonizers.
- C.albicans can cause oral thrush.
- The presence of coliforms or non-fermenting Gram negativebacilli indicates colonization, not pathogens in most cases. An exception could be in case of immunocompromised patients, where they can be reported "colonizers" in the comment column.
- Report as **normal flora** if only normal flora is found (alpha- hemolytic streptococcus, Gamma streptococci, non- pathogenic Neisseriae, Micrococci, diphteriodes, yeast etc).

8.IDENTIFICATION OF INDIVIDUALS ORGANISMS

Beta-*hemolytic streptococcus*

- Colonises are usually small, 1-2mm in size, smooth and glistening,with well-defined zones of beta-hemolysis. They are the commonest and are glossy colonises. Larger colonises, which are mucoid and watery, or colonises of average size, flattened, dried, and rough (matt or the post-mucoid colonises), may be seen occasionally. The matt colonises are rich in M protein and are more virulent than glossy colonises. Colonises of Group B streptococcus(GBS) are usually small,

grey, and somewhat centrally opaque, with hemolysis under the colony and with a narrow rim around the colony.

- Since Group A Streptococci (GAS) often lead to post-streptococcal sequelae, it is important to identify the antigenic groups of Beta hemolytic streptococci to institute pencillin prophylaxis at the earliest.
- Grouping of Beta hemolytic streptococci is done by either coagglutination or Latex agglutination.

10.GROUPING OF BETA HEMOLYTIC STREPTOCOCCI

a. Micro nitrous acid extraction method:

- Take 20 microlitre of 2M sodium nitrite solution in a test tube.
- Inoculate 3 to 4 colonies of Beta-hemolytic Streptococci into te test tube.
- Add 3 microlitre of glacial acetic acid to the suspension. Leave it at room temperature for 15 minutes.
- Add 16 to 24 microgram of sodium bicarbonate ($NaHCO_3$) for neutralization. Add 100microlitre of distilled water.
- Extract is now ready for CoA.

b.Broth suspension:

- Inoculate multiple colonies of beta-hemolytic streptococci into 1.0 ml Todd Herwitt Broth. Inoculate for 4-6 hours at 37oC. If enough growt had occurred to give a visible turbidity, proceed for co-agglutination grouping; otherwise continue incubation overnight.

c. Co-agglutination method.

- This method makes use of the unique property of Staphylococcus ayreus protein A to bind to the 'Fe' portion of IgG molecules,leaving 'Fab" portion free for the specific antigen binding. Usually broth culture of streptococci are used as antigens and positive results depend on their co-agglutionation by the antibody coated staphylococcal cells, when tested on a ring or cavity slide.

d.Preparation of Staphylococcal reagent.

- The Cowan I strain of S. aureus is grown in Todd Hewitt with 1% glucose overnight with continuous stirring using a magnetic stirrer.
- Centrifuge and wash the seiment five times using 0.03M PBS. Formalanise by adding 1.2ml of 40% formaldehyde to 100ml PBS.
- Keep for 3 hours at room temperature. Shake once or twice in between. Wash three times following this. Formalinized cells are heated in a water bath at 56^0C for 30 minutes to remove the foul odour.
- After 3 furthur washings the cells are suspended to 10% in PBS with 0.1% sodium azide as preservative. Suspension is stained using 1% methylene blue and Gram's iodine.

e.Sensitisation of Staphylococcal reagent.

- For sensitization 0.1ml of the group specific streptococcal antiserum is added to 1.0ml of the staphylococcal reagent, mixed and left at room temperature for 45 minutes.

- After centrifuging once, the sediment is resuspended in 10ml of PBS wit 0.1% sodium azide and stored at 4^0C.
- Antisera that do not cross react with heterologous group extracts by the capillary precipitation method may however show cross-reaction in the c0-agglutination method.
- Such cross-reactions can be eliminated by diluting the antiserum used for sensitization by absorption with cross-reacting cells, prior to sensitisation .
- Preparation of antigen for CoA antigen extracted by micronitrous acid method or broth culture as such may be used.

f. Technique.

- Place one drop of well-mixed broth culture or micro nitrous acid extract into rings cavities of the slide using Pasteur pipette.
- Add one drop of sensitized staphylococcal reagent (A,B,C & G) to each ring/cavity.
- Mix by rotation for about 3 minutes.
- Observe the mixture for evidence of agglutination, against diffused light.
- Grade results of agglutination as- 1+,2+,3+, &4+ depending on the degree of clumping and clearing observed.
- The group reagent, which gives the strongest agglutination, denotes the group of the streptococcal isolate.

SOP FOR ANAEROBIC MICROBIOLOGY

1.PURPOSE

To describe in detail the procedure followed for transport and processing of specimen from various sources, including pus, tissue samples, body fluids and blood for detection of anaerobic organisms.

2. HINTS FOR SELECTION OF SPECIMENS TO OPTIMIZE YIELD OF ANAEROBES:

1. Certain hints are useful to direct the clinician with regard to selection of specimen for anaerobic culture.

- Foul-smelling discharge.
- Presence of necrotic tissue and gangrene.
- Infection associated wit malignancy or other processes producing tissue destruction.
- Septic thrombophlebitis.
- Lack of response to the usual antibiotics.
- Repeatedly negative anaerobic blood culture.
- Presence of "sulphur" granules in discharges'
- Uterine discharge especially after childbirth and abortion when there is a history of interference and also pelvic inflammatory disease.
- Deep seated abscess.

3.SPECIMEN.

- Blood, body fluids, pus & tissues from various sources received in sterile test tube or screw capped bottles.

- Purulent material or tissue sent in a sterile test tube is the specimen of choice. Swab specimen are to be avoided as the yield is very poor.
- NOTE: For diagnosis of gas gangrene:

➢ Necrotic muscle tissue (ideal specimen)

➢ The next choice, fluid aspirated from oedematous tissue.

- Actinomycosis: The gauze dressings containing the granules are the most satisfactory specimens.

Cardinal points in the study of anaerobic bacteria which enhance yield of anaerobes:

- Most important: Prompt transport and inoculation of media
- Gram-stained smears of all materials received including wound tissue, but excluding blood, must be made and examined at once.
- Always use fresh media
- Proper anaerobic conditions should be provided.
- Prompt and processing minimizing exposure to ambient atmosphere.
- Usage of leak free anaerobic jars
- Ensuring proper anaerobiosis, complete evacuation and replacement with gas in proper proportion and duration.
- Subculture of colonies immediately after removal from an anerobic environment.

4.PROCESSING

a.Purpose

To describe in detail the microscopic examination, culture of anaerobic pathogens isolated from blood, body fluids, pus from various sources and tissue samples.

b. Making Gram-stain smears and examine.

Look for organisms as indicated below:

1. Pale, irregularly staining, pleomorphic slender Gram-negative bacilli (Bacteriodes)
2. Gram-negative rods, long and slender, with tapered ends (Fusobacterium).
3. Large, branching filamentous, Gram-positive organisms or bacilli (Actinomyces).
4. Thin, branching filamentous, Gram- positive organisms or bacilli (Actinomyces).
5. Small Gram-positive cocci (Peptostreptococcus)
6. Small Gram- negative cocci in pairs or groups, (Veillonella).

c. Culture.

c.1.Primary culture.

- Inoculate primary isolation media soon after receipt of specimen.
- Use freshly prepared, adequately dried, agar plates.

- Heat fluid media in a boiling water for 10 minutes just before inoculation to drive out the dissolved oxygen.

c.2.The media routinely inoculated for isolation of anerobes.

- Blood agar (BA) without base used for anaerobic culture (ANBA).
- Neomycin Blood agar (NBA)
- Thioglycollate Broth
- Robertson's cooked Meat medium (RCM)
- Extra plates must be added if the following organisms are suspended:

Clostridium species

- 4% Blood agar.

  Egg Yolk agar (EYA)

  Willis and Hobbs medium(W&H) (optional).

NOTE: For isolation of C.tetani, other vegetative forms need to be destroyed. Hence, the material is heated at 85^0C for 10 minutes and then inoculated into RCM.

Provotella species/ Porphyromonas species

- Include Lysed Blood Agar (LBA)

Actinomycetes

- Brain Heart Infusion Agar (BHIA),ANBA and two or three Thio's to be inoculated depending on amount of material available.

Choose appropriate anaerobic system and follow directions given below:

5. METHOD FOR ACHIEVING ANAEROBIOSIS IN JARS:

Evacuation- Replacement System

- Place the inoculated plates inside the jar, media facing upwards.
- Evacuate the jar using vaccum pump.
- Fill the jar with hydrogen from a cylinder.
- Using a vaccum pump evacuate the hydrogen from the jar.
- Introduce hydrogen and $c0_2$,likewise, from cylinders, in a ratio of 90% H_2:10%Co_2 using a pressure gauge as monitor.
- NOTE:

➢ Gas-pak system (is used in the lab when no Co_2/H_2 is available or when the vaccum pump is under repair)

➢ Use a commercially available automatic hydrogen and carbon dioxide "generator" contained inside a packet.

➢ Attach a balloon to the jar for collection of the displaced air.

➢ Place the inoculated plates inside the jar.

➢ Open the Gas-pak at one corner, place it inside the jar; add about 10ml of distilled water.

➢ Secure the lid of the jar immediately.

- When the balloon distends completely close the outlet, and place the jar in the incubator.

QUALITY CONTROL

- An indicator methylene blue may be used (anaerobic- colourless,exposure to O2-blue)
- C.*perfrigens* & B.*fragilis*- always included in each jar.
- New jar- include strict aerobe(Ps.*aeruginosa*) & strict anaerobes (C. *tetani*)

Anoxomat Mark II- cultivation of anaerobic, microaeropillic and capnopillic bacteria:

In our laboratory since Jan 2012- the Evacuation- replacement systems has been preformed by fully automated "Anoxomat" Mark II system. This is manufactured from Netherland. By this method we can cultivate anaerobic, microaerophilic and capnopilic bacteria

- Anaerobic- with 80% N2,10% H2 and CO2

This system is connected with three types of gas cylinders:

1. Mixed gases (N2, H2 & Co2)
2. CO2- Carbondioxide
3. Nitrogen.

All the accessories are provided by the manufacturer.

- Jars
- The clamp

- The quick (snap-shut) coupling
- Catalyst-(palladium coated aluminium pellets)
- Halamid jar cleaner disinfectant.

Jar:

The jar is made up of polymethylmethacrylate (PMMA)

It has to be cleaned with the- disinfectant Halamid just before use.

Halamid:

It is based on latent chlorine and O2.

Effective against bacteria, fungi and viruses.

Not aggressive towards metals and other materials such as rubber, plastics and wood. We are using 0.5% Halamid.

Procedure:

- Before use clean the jar with Halamid.
- Place all the plates to be incubated with catalyst inside the jar.
- Place the lid and clamp it tight.
- Connect the jar with Anoxomat machine using the coupling.
- Switch on the machine and open the gas valve for three times.
- Select the needed options (anaerobic/ microaerophilic).
- Switch on the green button.

- The processing begins- gas pressure should be between 1.6- 1.7 Bar.
- The Anoxomat evacuates a portion of the jar contents and refills the jar with an anaerobic gas mixture.
- During this procedure the oxygen in the air is rarefied.
- In case of an anaerobic recipe, this procedure is repeated three times, after which the oxygen concentration is rarefied to 0.16%. A small catalyst removes this very small percentage.
- The automated system itself performs the quality assurance before incubation.

➢ Double jar- leak test.

➢ Gas input test.

➢ Catalyst activity test-120 sec.

- After all the are ok it shows signal and finally the screen shows finished.
- Switch off the green button, close the gas valve and switch off the machine.

6.**Incubation**

- Incubate TB and RCM at 37^{o}C aerobically.
- Keep the jars in the incubator (37^{o}C).
- Incubate cultures for a minimum period of 72 hours at 37^{o}C except as indicated below.
- Incubate cultures for fastidious anaerobes for a longer period.
- Shorten the incubation period to 24 hours for gas gangrene specimens where the presence of C.*perfringens* is suspected from direct smear findings.

- In the case of actinomycos*is*, examine after 36-48 hours incubation for growth, but hold for 7-10 days for development of 'molar tooth' colonies, if *A.israelii* is suspected.
- NOTE: Proceed to do anaerobic methodology also for specimens on which only aerobic culture is requested in the following instances, even in the absence of a request for such.
- If organisms seen in direct smears fail to grow aerobically.
- If growth occurs in "anaerobic zone" of TB, smear shows organism which fail to grow on aerobic subculture.

7.SUBCULTURE

- Subculture TB and RCM after 72 hours of incubation; onto BA and NBA and special media also, if necessary. Place plates inside an anaerobic jar for incubation.
- After 72 hours of anaerobic incubation examine all primary as well as subculture plates for evidence of growth.
- Note colony morphology carefully. Make and examine Gram-stained smears to determine cellular morphology.
- Check for "Aero tolerance" by inoculating BA plates and incubating in ordinary atmosphere at 37^{o}C.

NOTE:

- The jar used in this laboratory are metal jar (BT jar) and Transparent PMMA- poly methyl methacrylate.
- The Pallaium coated aluminium pellets covered with wire mesh- Cold catalyst used is active at room temperature.
- The cold catalyst can be rejuvenated by heated in a hot air oven at 160^{o}C for 1 hour & stored in a closed container.

8.IDENTIFICATION

- Once pure growth has been obtained, inoculate media to determine biochemical reactions, and for carry out pertinent special tests.

8.a.Blood for anaerobic culture.

BacT/ ALERT FA culture bottles are used with the BacT/ALERT Microbial Detection System in qualitative procedures for enhanced recovery and detection of anaerobic microorganisms from blood. The BacT/ALERT Microbial Detection System is used to determine if microorganisms are present in blood taken from a patient suspected of having bacteremia. The BacT/ALERT System and culture bottles provide both a microbial detection system and a culture media with suitable nutritional and environment conditions for organism commonly encountered in blood infections. An inoculated bottle is placed into the instrument where it is incubated and continuously monitored for the presence of microorganisms that will grow in the BacT/ALERT FA bottle.

Principle of the test:

The BacT/ALERT Microbial Detection System utilizes colorimetric sensor and reflected light to monitor the presence and production of Co_2 dissolved in the culture medium. If microorganisms are present in the test sample, Co2 is produced as the organisms metabolise the substrate in the culture medium. When growth of the microorganisms produce CO_2 , the colour of the gas-permeable sensor installed in the bottom of each culture bottle changes from blue-green to yellow. The lighter color results in an increase of reflectance units monitored by the system. Bottle reflectance is monitored and recorded by the instrument every 10 minutes.

After bottles have been loaded into the instrument, incubate 5 to 7 days or until designated positive. Smear is made from bottles, designed positive. Anaerobic subculture is done and proceeded for identification of the isolate, as for other clinical samples.

QUALITY CONTROL STRAINS FOR CHECKING MEDIA, BIOCEMICALS AND ANAEROBIC JAR.

Anaerobic blood agar

Neomycin blood agar

Thioglycollate

ATCC13124 Clostridium perfringens

ATCC25285 Bacteroides fragilis

Robertson's cooked meat media

4% Blood agar	-	ATCC11437 Clostiridium sporogenes & Clostridium tetani
Lysed Blood agar	-	Prevotella melaninogenica & Porphyomonas asacchrolytica

Other strains:

ATCC9689 Clostridium difficle

Fusobacterium spp

Actinomyces israelli

Anaerobic gram positive cocci.

BIOCHEMICAL TESTS FOR IDENTIFICATION:

1. **Bateriodes and other GNB**

BA: medium sized, grey, smooth non-haemolytic colonies

Colony: Pleomorphic small negative bacilli

Species differentiation is based on biochemical reactions.

Refer to below for identification scheme for *Bacteriodes, Porphyromonas, Prevotella*

Biochemical reaction of Porphyromonas and Prevotella spp.

BIOCHEMIAL REACTION OF COMMONLY ENCOUNTERED *Bacteriodes* spp.

TEST	B.fragilis	B.distasionis	B.vulgaris	B.ovatus	B.thetaiotaomicron
Indole	negative	negative	negative	positive	positive
Rhamnote	negative	variable	positive	positive	positive
Mannite	negative	negative	negative	positive	negative
Trehalose	negative	positive	negative	positive	positive

TEST	Prevotella melaninogenica	Prevotella intermidia	Porphyromononas saccharolytica
Esculin	Variable	negative	negative
Glucose	positive	positive	negative
Starch	positive	Positive/negative	negative
Lactose	positive	negative	negative
Indole	negative	positive	positive
Gelatine	Varible	positive	Varible
Trahalose	positive	Varible	negative

FUSOBACTERIUM

1. BA: characteristic greening observed
2. Colony smear: long, thin, Gram-negative bacilli with tapering ends
3. Biochemically inert ,indole test is usually positive.

- Susceptible to pencillin
- Growth is inhibited by 20% bile.

Species differentiation is based on biochemical reactions as given in the table below:

BIOCHEMICAL REACTIONS OF *Fusobacterium* species

TEST	F.mortiferum	F.nucleatum	F.varium	F.necrophorum
Indole	negative	positive	variable	positive
Esculin	positive	negative	negative	negative
Lipase	negative	negative	negative	positive
Glucose	positive	variable	negative	variable
Lactose	variable	negative	negative	negative
Gelatin	negative	negative	negative	Positive/negative

CLOSTRIDIA

Gram stain appearance of clostridia

Clostridia spp	Gram reaction and	Spore morphology

	morphology	
C.Perfringens	Gram-positive, thick bacilli,with square ends	No spores
C.tetani	Gram-variable bacilliary forms	Terminal, sphereical,bulging spores (drum stick)
C.difficile	Gram-positive bacilli	Oval, sub-terminal non-bulging spores
C.sporogenes	Gram-positive bacillus	Oval, central or sub terminal bulging spores
C.novyi (Type A)	Gram-positive bacillus	Oval, central or sub terminal bulging spores
C.botulinum	Gram-positive bacillus	Oval, central or sub terminal bulging spores

Culture characteristics of Clostridium spp.

Clostriduim spp	BA	EYA	RCM
C.Perfringens	Target hemolysis**	Opalescence*	Saccharolytic
C.tetani	Spreading film of growth, non-hemolytic	Spreading film of growth	Weakly proteolytic
C.difficile	NH irregular (Horse	No opalescence	Saccharolytic

	dung odour)		
C.sporogenes	Beta-hemolytic,irregular edge	Pearly layer	Proteolytic and saccharolytic
C.novyi (Type A)	Target hemolysis	Opalescenece and pearly layer	Saccharolytic and weakly proteolytic.
C.botulinum	hemolytic,irregular edge	Opalescenece and pearly layer	Proteolytic

** Target haemolysis on BA

On BA, the colonies are discrete, surrounded by a clear zone of beta- hemolysis, which again is surrounded by a hazy zone of hemolysis, is called the “target haemolysis”

Reverse CAMP test

Procedure

1. On to a TSBA/BA plate (it may be divided into three sections and used). Streak C.perfrigens as a straight line.

2. Cross streak GBS at right angles to this in the middle, taking care not to touch the C.perfringens streak
3. Include positive and negative controls.
4. Incubate in the anaerobic jar.
5. Read after over night incubation.
6. Presence of "butterfly-shaped" synergistic haemolysis constitutes a positive reaction.
7. Species differentiation is based on biochemical reaction

BIOCHEMICAL REACTIONS FOR SPECIES DIFFERENTIATION OF Clostridium spp.

Test	C.Perfringens	C.tetani	C.difficile	C.sporogenes
Esculin	V	-	+	+
Lecithinase	+	-	-	-/+
Lipase	-	-/+	-	+
Glucose	+	-	+	+
Litmus milk	V	-	-	+
Gelatin	+	+	-	+
Mannitol	-	-	+	-
Lactose	+	-	-	-
Indole	-	+/-	-	-

- = negative, + = positive, V = variable

8. Opalescence and pearly layer formation on EYA

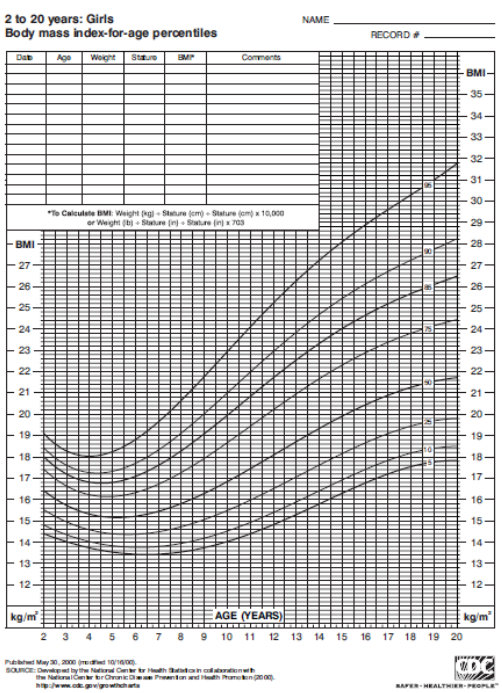
9. Lactose fermentation and proteolysis on W & H

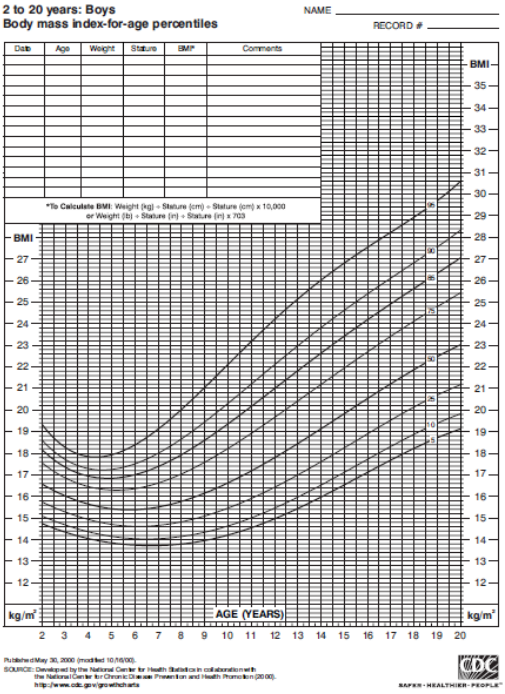
10. Nagler's reaction of opalescence on EYA and its inhibition

- Spread 1 loopful of gas-gangrene antitoxin over one half an EYA plate.
- Allow to dry
- Inoculate the organism to be identified by streaking a line starting from the side without antitoxin
- Incubate anaerobically at 37^0C.
- Read after 24-48 hours.
- Observation of tongue shaped opalescence in the absence of antitoxin and inhibition of opalescence in the presence of antitoxin is a positive test

10. Stormy clot in litmus milk.

GRAPH TO CALCULATE BMI IN CHILDREN ACCORDING TO THEIR AGE





EXCEL SHEET DATA

tonsillitis	age	sex	BMI	SES	Snore	T-grade	A-grade	PATHOGEN	S.aure	Ahs	Npn	Mrsa
1	6	2	12.8	2	0	3	2	NF	1	3	3	0
1	11	1	17.9	3	0	3	2	HPARA(3)	2	3	2	0
1	7	1	17.2	3	1	3	3	NF	1	3	2	0
1	9	2	15.4	3	1	3	4	NF	0	2	1	0
1	4	1	14.5	4	1	3	3	NF	0	0	0	1
1	6	1	13.4	3	1	4	4	NF	0	1	1	0
1	6	1	12.4	2	0	3	1	GBS(3)	2	3	0	0
1	13	2	21.4	2	0	3	3	NF	0	3	1	0
1	4	2	12.4	4	1	3	2	S.PNEU(3)	0	3	3	2
1	6	1	13.4	4	1	3	3	NF	0	3	2	0
1	5	1	16.3	2	1	2	1	HPARA(3)	0	3	2	0
1	6	1	14.9	3	0	3	3	GABHS(3),HINF(3)	0	3	2	0
1	10	1	17	2	1	2	A3C2E1	NF	2	1	2	0
1	5	1	14.6	2	1	4	ND	NF	0	2	2	2
1	10	1	24.2	4	1	4	2	HPARA(3)	3	3	3	0
1	6	1	15.3	2	1	3	3	HPARA(3)	2	2	3	0
1	14	1	13.5	2	1	3	1	GGBHS(3),HPARA(3)	3	1	0	0
1	6	2	20.1	3	1	3	A3C2E1	NF	3	0	1	0
1	5	2	15.2	2	1	4	4	S.PNEU(1)	0	1	1	0
1	6	1	20.3	3	1	3	4	NF	0	1	1	0
1	6	1	14.4	2	0	3	2	S.PNEU(2)	1	1	0	0
1	5	1	13.3	3	0	2	1	HPARA(3),S.PNEU(2)	0	1	1	0
1	5	2	14.9	2	1	3	3	NF	0	0	1	0
1	14	1	28	5	0	3	ND	NF	1	1	0	0
1	5	1	13.4	4	1	3	3	GGBHS(3)	0	3	3	0
1	5	2	15.2	4	1	2	4	HINF(3),GFBHS(3)	0	3	3	0
1	4	1	13.4	3	0	3	ND	HINF(3)	0	2	2	0
1	4	1	15.4	4	1	2	4	HINF(2)	0	1	1	0
1	7	1	18.9	4	1	3	3	HINF(3)	0	3	0	0
1	9	2	17	3	1	3	3	HPARA(3)	0	3	3	0
1	9	2	18.1	3	0	2	2	NF	1	1	1	0
1	14	1	25.1	5	0	3	3	HINF(3)	0	3	3	0
1	5	1	11.3	3	0	3	3	NF	0	2	0	0
1	9	1	17	4	0	2	2	HPARA(3)	3	3	3	0
1	4	2	13.2	3	1	3	4	NF	1	2	1	0
1	9	1	23	3	0	3	2	HPARA(3)	0	3	3	0
1	8	2	12.5	3	1	3	4	GGBHS(2),HPARA(2)	0	3	3	0
1	6	2	14.4	3	1	3	4	KLEB(1)	0	3	2	0
1	7	1	18.3	4	0	3	2	HPARA(1)	0	1	1	0

1	5	2	13.6	3	0	3	ND	HINF(3)	0	3	3	0
0	6	1	16.5	3	NA	1	NA	GGBHS(3)	0	3	3	0
0	5	1	19.8	3	NA	1	NA	HPARA(3)	0	3	2	0
0	10	1	15.4	2	NA	1	NA	HPARA(3)	0	3	1	0
0	5	2	13.4	2	NA	1	NA	HPARA(3)	0	3	3	0
0	7	2	11.7	2	NA	1	NA	NF	0	3	3	0
0	9	1	15.8	4	NA	1	NA	HPARA(3)	0	3	2	0
0	4	1	12.5	3	NA	1	NA	HPARA(3)	0	3	2	0
0	7	1	13.9	4	NA	1	NA	NF,PSEUO(3)	0	3	0	0
0	13	1	18.7	2	NA	1	NA	NF	0	2	2	0
0	5	2	14	3	NA	1	NA	HPARA(3)	0	1	1	0
0	10	1	16.1	3	NA	1	NA	NF	0	2	0	0
0	4	2	17.7	2	NA	1	NA	GABHS(2)	0	3	3	0
0	6	2	12.9	2	NA	1	NA	NF	0	3	3	0
0	11	2	19	3	NA	1	NA	NF	0	3	1	0
0	5	1	15.3	3	NA	1	NA	NF	0	2	2	0
0	9	1	38.4	3	NA	1	NA	NF	0	3	3	0
0	13	2	27.6	3	NA	1	NA	HPARA(3),GGBHS(2)	0	2	0	0
0	8	1	14.8	2	NA	1	NA	GGBHS(1),HPARA(3),S.PNEU(3)	0	2	0	0
0	8	1	15.6	3	NA	1	NA	GGBHS(3),HPARA(3)	0	3	3	0
0	11	1	19	3	NA	1	NA	NF	1	3	0	0
0	12	1	17.9	3	NA	1	NA	GGBHS(3),HPARA(3)	3	3	3	0
0	6	1	14.8	3	NA	1	NA	HPARA(3)	0	3	1	0
0	11	1	20.2	4	NA	1	NA	GGBS(3),HPARA(3)	0	3	0	0
0	11	2	19.4	2	NA	1	NA	HPARA(2)	0	3	2	0
0	12	1	14.5	3	NA	1	NA	HPARA(3)	2	3	3	0
0	10	2	14.4	3	NA	1	NA	NF	1	3	3	0
0	11	1	21.7	4	NA	1	NA	HPARA(3)	0	3	3	0
0	11	2	16.6	4	NA	1	NA	HPARA(3)	0	3	1	0
0	11	2	18.4	4	NA	1	NA	NF	0	3	3	0
0	5	1	15.4	5	NA	1	NA	GGBHS(3),HPARA(3)	0	3	2	0
0	7	2	16.7	2	NA	1	NA	NF	0	3	3	0
0	5	1	14.4	3	NA	1	NA	NF	0	3	3	0
0	13	2	20.7	3	NA	1	NA	NF	1	0	0	0
0	4	1	12.4	4	NA	1	NA	NF	0	3	3	0
0	12	1	14.6	4	NA	1	NA	GGBHS(3)	0	3	2	0
0	6	1	15.9	2	NA	1	NA	GABHS(1)	0	3	3	0
0	5	1	13.5	3	NA	1	NA	HPARA(2)	1	3	3	0
0	4	1	13.4	4	NA	1	NA	NF	1	3	2	0
0	10	1	17.5	3	NA	1	NA	NF	1	3	2	0
0	5	1	15.8	5	NA	1	NA	NF	1	3	2	0

Nf gnb	P.aeuro	Gbhs	Dipht	H.para	NG stre	CoNS	no	P.mag	F.nucl	P.sacc	F.var	B.fra	AGPC	Fuso	AGNB
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
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0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
0	0	0	0	0	3	0	0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	0
3	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
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0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
0	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
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0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
2	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
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0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0
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0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0