A PREVALENCE STUDY OF MICROBIOLOGICAL PROFILE IN PUS SAMPLES OF CHRONIC SUPPURATIVE OTITIS MEDIA PATIENTS, DRUG RESISTANCE PATTERN AND ITS MOLECULAR CHARACTERISATION AT THANJAVUR MEDICAL COLLEGE AND HOSPITAL, THANJAVUR.

Dissertation submitted to

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY.

In partial fulfilment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

BRANCH - IV

THANJAVUR MEDICAL COLLEGE

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

THANJAVUR – TAMILNADU

MAY 2020

CERTIFICATE

This is to certify that this dissertation titled **"A PREVALENCE STUDY OF MICROBIOLOGICAL PROFILE IN PUS SAMPLES OF CHRONIC SUPPURATIVE OTITIS MEDIA PATIENTS, DRUG RESISTANCE PATTERN AND ITS MOLECULAR CHARACTERISATION AT THANJAVUR MEDICAL COLLEGE AND HOSPITAL"** "is a bonafide record done by **Dr.B.SHANTHI BHUSHANA,** during the period of her Post graduate study from April 2018 to June 2019 under the guidance and supervision in the Institute of Microbiology, Thanjavur Medical College Hospital, Thanjavur, in partial fulfilment of the requirement for **M.D.MICROBIOLOGY** Degree Examination of the Tamilnadu Dr.M.G.R.Medical University to be held in May 2020.

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DECLARATION

I declare that the dissertation entitled **"A PREVALENCE STUDY OF MICROBIOLOGICAL PROFILE IN PUS SAMPLES OF CHRONIC SUPPURATIVE OTITIS MEDIA PATIENTS, DRUG RESISTANCE PATTERN AND ITS MOLECULAR CHARACTERISATION AT THANJAVUR MEDICAL COLLEGE AND HOSPITAL"** submitted by me for the degree of M.D. is the record work carried out by me during the period of April 2018 to June 2019 under the guidance of **DR. EUNICE SWARNA JACOB,** Head of the Department of Microbiology, Thanjavur Medical College, Thanjavur. This dissertation is submitted to the Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfilment of the University regulations for the award of the degree of M.D. Microbiology (Branch Ⅳ) Examination to be held in May 2020.

Place: Thanjavur, \

Date: (Dr. B. SHANTHI BHUSHANA)

ACKNOWLEDGEMENT

First and foremost I thank the Almighty, for giving me health and strength for being with me and guide me throughout the preparation of this dissertation and its successful completion.

I wish to express my sincere thanks to our **Dean Dr.KUMUDHA LINGARAJ** for permitting me to use all the available material resources of this institution during the period of my study.

Our Head of the Department **Dr. EUNICE SWARNA JACOB** needs special mention. She energised me at every step as a catalyst to draw out the best from me. I profoundly thank her for her immense help.

My sincere thanks to Prof**. Dr. BALASUBRAMANIYAN**, Department of Ear, Nose and Throat, Thanjavur Medical College Hospital for permitting me to conduct the study, and having been so kind to provide me with all data available in the department.

I whole heartedly thank my Guide Senior Assistant Professor **Dr. AYISHA** who guided me from the selection of the topic to the submission of the dissertation guided me and gave me great support throughout my study.

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PREVALENCE STUDY OF MICROBIOLOGICAL PROFILE IN PUS SAMPLE OF CSOM, DRUG RESISTANCE PATTERN AND ITS MOLECULAR CHARACTERISATION AT THANJAVUR MEDICAL COLLEGE & HOSPITAL

submitted by Dr. B. SHANTHI BHUSHANA **of**

Dept. of MICROBIOLOGY **Thanjavur Medical College, Thanjavur**

was approved by the Ethical Committee.

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TABLE OF CONTENTS

INTRODUCTION

WHO defines Chronic suppurative otitis media as a "stage of ear disease in which there is chronic infection of middle ear cleft, perforated ear drum (non-intact tympanic membrane) and discharge (otorrhea) for at least preceding two weeks" [8]

CSOM is defined as persistent or intermittent infection of ear for more than three months duration where the infected discharge comes through the perforated tympanic membrane caused by bacteria, fungi and virus resulting in inflammation of mucosal lining that results in partial or total loss of the tympanic membrane and ossicles.[14]

Prevalence of CSOM is more in the developing and underdeveloped countries because of poor hygiene practices, overcrowding, malnutrition, inadequate health care and recurrent URTI.[9]

CSOM can be classified as non cholesteatomatous chronic otitis media with perforation of the tympanum and chronic cholesteatomatous otitis media regardless of eardrum perforation.[11]

CSOM is the most common chronic disease seen especially in infants and children causing conductive deafness which may lead to delayed development of speech and language in children. Untreated cases of CSOM can result in various complications.[10]

Such complications range from persistent otorrhea, mastoiditis, labyrinthitis and facial nerve paralysis to serious complications like meningitis, intracranial abscesses and thrombosis.[10]

CSOM is a major public health problem and India is one of the countries with highest CSOM prevalence where urgent attention is needed (WHO, 2004)[12]

The most predominant organisms causing CSOM among aerobic bacteria are Pseudomonas aeruginosa followed by Staphylococcus aureus. Of the fungal isolates Aspergillus species are commonest followed by Candida species.[10] Knowledge of microorganisms commonly associated with CSOM and their drug susceptibility pattern will contribute to appropriate antibiotic usage and successful treatment. Haphazard use of antibiotics and increasing use of newer one has led to persistent change in microbial flora. [13]

The purpose of this study was to identify the common microbial pathogens causing CSOM and to determine their antimicrobial sensitivity pattern and resistance pattern.

AIMS AND OBJECTIVES

1. To isolate and identify aerobic bacteria and fungi causing CSOM patients.
2. To study the prevalence of CSOM in our hospital.
3. To determine antimicrobial sensitivity and resistant pattern of the bacterial isolates in CSOM patients.
4. To study the molecular characterisation of commonest bacterial isolate by polymerase chain reaction.

REVIEW OF LITERATURE

Chronic suppurative otitis media is a persistent inflammation of middle ear of mastoid cavity. It is characterised by persistent or recurrent ear discharge over 2-6 weeks through a tympanic membrane perforation. Additional features are thickened granular middle ear mucosa, mucosal polyp and cholesteatoma within the middle ear.

ANATOMY OF EAR:

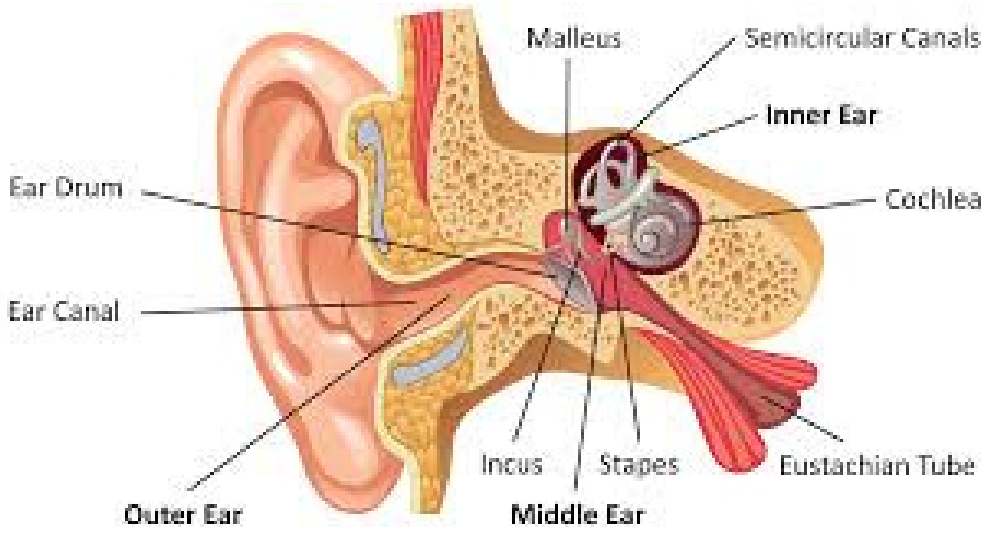

FIG 1: Anatomy of Ear

The ear is an organ of hearing and maintaining the equilibrium of the body. It consists of three parts. The external ear, the middle ear and the internal ear.

The middle ear is also called the tympanic cavity or tympanum.

Parts:

1. Tympanic cavity proper-opposite to tympanic membrane
2. Epitympanic recess- above the level of the tympanic membrane

Communications:

It communicates anteriorly with the nasopharynx through the auditory tube, and posteriorly with the mastoid antrum and mastoid air cells through the aditus to the mastoid antrum.

Contents:

1. Three small bones or ossicles namely the malleus, the incus and the stapes.
2. Ligaments of the ear ossicles.
3. Two muscles, namely the tensor tympani and the stapedius.
4. Vessels supplying and draining the middle ear.
5. Nerves, tympanic plexus and chorda tympani nerve.
6. Air

Boundaries:

Roof or Tegmental wall:

1. It is formed by a thin plate of bone called the tegmen tympani. This bone is prolonged backwards as the roof of the canal for the tensor tympani.
2. The roof separates the middle ear from the middle cranial fossa.

Floor or Jugular wall:

1. This plate is a part of the temporal bone.
2. It separates the middle ear from the superior bulb of the internal jugular vein.

Anterior or Carotid wall:

1. The uppermost part of the anterior wall bears the opening of the canal for the tensor tympani.
2. The middle part has the opening of the auditory tube.
3. The inferior part of the wall is formed by a thin plate of bone which forms the posterior wall of the carotid canal. This plate separates the middle ear from internal carotid artery.
4. The bony septum between the canals for the tensor tympani and for the auditory tube is continued posteriorly on the medial wall as a curved lamina called the Processes cochleariformis.

Posterior or mastoid wall:

1. Superiorly, there is an **aditus** through which epitympanic recess communicates with the mastoid antrum.
2. **Fossa incudis** is a depression which lodges the short process of incus.
3. **Pyramid,** lies near the junction of the posterior and medial walls.
4. **Posterior canaliculus** through which the chorda tympani nerve enters into the middle ear cavity.

Lateral or membranous wall:

1. It is formed mainly by the tympanic membrane and partly by the squamous temporal bone.

Medial or Labyrinthine wall:

1. The **promontory** is produced by the first turn of cochlea.
2. The **fenestra vestibuli** is closed by the foot-plate of stapes.
3. The **prominence of the facial canal** runs backwards just above the fenestra vestibuli.
4. The **fenestra cochleae** is closed by the secondary tympanic membrane.
5. The **sinus tympani** is a depression behind the promontory.

Risk factors:

- Multiple episodes of acute otitis media.
- Frequent upper respiratory tract infection, nasal disease.
- Poor living conditions.

- Attending congested day care centre.
- Bottle feeding.
- Inadequate antibiotic treatment.
- Family history of otitis media.

Classification:[61]

- Anatomical
- Pathological
- Clinical

I. **Anatomical:**

1. Tubotympanic disease

It is characterised by perforation in pars tensa. They are not developing serious complications generally. Hence the term "safe ear" used. It is unassociated with cholesteatoma. It occurs due to the sequel of acute otitis media or an ascending infection from nasopharynx through the eustachian tube. It is called safe or benign type of disease because there is no danger to the life of the patient.

The disease is confined to the anteroinferior part of middle ear and mucosa of eustachian tube.

Pathogenesis:

- ✓ Chronic inflammation of middle ear mucosa
- ✓ Perforation in pars tensa part of tympanic membrane

- ✓ Tympanosclerosis – it is hyalinisation and subsequent calcification of the subepithelial connective tissue present in the remnants of tympanic membrane, ossicles, tendons, oval window and round window

Clinical features

Discharge from ear: serous or mucoid or frankly purulent in nature and continuous or intermittent

Hard of hearing: In majority of the cases, it is conductive type of hearing loss. Factors contributing conductive hearing loss include size of perforation, impairment of ossicles and middle ear pathology like oedema and granulation tissue.

Complications:

Otitis externa, Ossicular chain erosions, hearing loss, vertigo, tympanosclerosis and adhesions

2.Atticoantral disease

It involves pars flaccida commonly. It is characterised by formation of a retraction pocket in which keratin accumulate to produce cholesteatoma. It is an active squamous disease in which squamous epithelium present in the middle ear cleft erodes the bone.

It is also called unsafe or dangerous ear because of developing serious complications

Predisposing factors:

Due to repeated infections, middle ear mucosa undergoes metaplastic changes and converts into squamous epithelium. Cholesteatoma keratinising squamous epithelium replaces the ciliated columnar epithelium of the middle ear.

Pathogenesis:

It includes,

- ✓ Granulation tissue with keratin masses providing a good support to growth of bacteria.
- ✓ Ossicular chain necrosis and cholesterol granuloma.
- ✓ Due to negative intratympanic pressure, there is a formation of retraction pocket in the attic region.

Clinical features:

Symptoms: ear discharge usually foul-smelling scanty discharge due to bone destruction. Sometimes blood-stained discharge indicates presence of osteitis and granulation tissue.

Signs: Perforation, retraction pocket and cholesteatoma

Complications:

It includes,

1. Extracranial – meningitis, brain abscess, lateral sinus thrombosis, otitic hydrocephalus, extradural and subdural abscess.

2. Intracranial – mastoiditis, petrositis, labyrinthitis and facial paralysis

Ⅱ Pathological:

✓ **Inactive chronic otitis media:**

There is a permanent defect in pars tensa, but there is no inflammation either tympanic membrane or middle ear mucosa.

✓ **Active chronic otitis media:**

There is a middle ear mucosa inflammation and is characterised by edema, excess mucus production. In some other cases, granulations or polyp may develop. Tympanic membrane perforation is also present

✓ **Active squamous epithelial chronic otitis media Cholesteatoma**

In addition to the active mucosal otitis, there is squamous epithelial lined pocket full of squamous epithelial and inflammatory debris. This is most commonly occur in pars flaccida than pars tensa.

✓ **Inactive squamous epithelial chronic otitis media Retraction pocket**

It is the pars flaccid or pars tensa retraction, having the potential to retain squamous debris, which may lead to cholesteatoma.

Ⅲ Clinical:

Thorburn (1965) described tubotympanic disease into two types.

✓ Permanent perforation syndrome (LILLIE type 1) This is characterised by permanent perforation of tympanic membrane, involving pars tensa. The ear may be completely dry for a longer period or it may discharge

intermittently. In the dry state, tympanic membrane is pink and debris is not present in the tympanum.

- ✓ Chronic tubotympanic mucositis (LILLIE type 2) This infection is characterised by prolonged duration, profuse mucopurulent discharge, large or near total defect of tympanic membrane and is associated with upper respiratory tract infection. The mucosa on the promontory is markedly thickened and red. Polyps may be present as a result of marked swelling of this oedematous mucosa. The ossicles are buried in this oedematous mucosa. Polyps may be associated with necrosis of ossicles.
- ✓ In some cases, after a long-standing suppuration, there is a growth of epithelium around perforated margins leading to secondary cholesteatoma. This type of cases are classified under Lillie type 3.

Types of perforations:

According to their anatomical location in relation to their handle of malleus they can be classified as anterior, posterior and inferior.

Central perforation – defect in pars tensa and are surrounded by residual tympanic membrane.

Attic perforation – located in pars flaccida.

Subtotal perforation – large defect is surrounded by intact annulus.

Marginal perforation – characterised by pathological loss of annulus exposing bony canal wall. Usual location is in the tympanic membrane's posterior part.

PATHOLOGY

Active mucosal disease:

Non secretory middle ear mucosa is replaced by respiratory mucosa with goblet cells. Mastoid mucosa seldom undergoes metaplasia. Mucosa is hyperaemic with underlying inflammatory response.

Inactive chronic otitis media:

Consistent finding is the loss of fibrous layer. When perforation is present outer squamous epithelium reaches the middle ear mucosa at variable points.

Etiology:

- ✓ Genetic: The size of the mastoid air cell system is inversely proportional to the occurrence of otitis media.
- ✓ Environmental: Lower socioeconomic status groups having higher incidence of developing CSOM. It is related to poor nutrition, overcrowding and general ill health.
- ✓ Infective:[63] Bacteria are secondary invaders of mucosa which is inflammed due to some other factors.

- ✓ Previous acute otitis media:[62] In most of the cases, CSOM occurs as a result of an episode of perforated ASOM with subsequent failure of perforation to heal.
- ✓ Upper respiratory tract infection: In majority of the cases, their ear starts to discharge after an URTI. A postulate is that any part of the respiratory mucosa is infected, there is an increased chance of developing infection in another part.
- ✓ Allergy: Allergic individuals have higher chance of developing CSOM.
- ✓ Autoimmunity:[64] There is an increased likelihood that individuals with established autoimmune disease will have higher incidence of CSOM, but only rheumatoid arthritis been proved till date (**Camilleri et al**1992)
- ✓ Trauma: Traumatic perforation and following grommet insertion (iatrogenic) may predispose to develop CSOM.
- ✓ Eustachian tube abnormality: anatomical defect in eustachian tube (e.g cleft palate), eustachian tube malfunction (e.g patulous eustachian tube) predispose to CSOM.

Investigations:

- History: unilateral or bilateral, painless otorrhea, deafness.
- Otoscopic examination: gives detail about presence of granulation tissue, keratin debris, perforation edges, adhesions and tympanosclerosis, extent of the defect and ossicular chain involvement.

- Audiometry: it gives an assessment of degree of hearing loss.
- Microbiological investigations:

To do Pus culture and sensitivity – this is to isolate the pathogens and its antimicrobial sensitivity pattern.

Specimen collection and transport: Swabs suitable for taking specimens of exudate from ear are sterile cotton swab or synthetic fibre, mounted on a thin wire or stick. Time relapse before processing the sample should not be more than 2hrs. The specimen can be stored in refrigerator at 2-8℃

Direct gram stain: To predict the likely pathogens. Report the gram stain finding as an initial report. Direct gram stain shows,

- Pus cells
- Epithelial cells
- Gram positive cocci or gram-negative bacilli
- Gram positive budding yeast cells.

Ear specimen should be inoculated to nutrient agar, blood agar, MacConkey agar and sabouraud dextrose agar. Furthermore, perform biochemical reactions and antimicrobial sensitivity pattern.

- Tuning fork test: To differentiate whether it is a nerve deafness or conductive deafness. The tests are based on the principle that normally aerial conduction of sound is better than bony conduction. In conductive

deafness, bony conduction becomes better than aerial conduction. In nerve deafness, both type of conductions are lost.

In **Rinne's test**, a vibrating tuning fork is held opposite the ear. When one stops hearing the sound, it is then placed on the mastoid process. The patient is asked to compare the relative loudness of the fork in the two instances.

In **Weber's test**, the vibrating tuning fork is placed on the centre of the forehead. The fork is heard better on the side of middle ear disease than on the normal side.

- X ray mastoid: To identify sclerosis and bony erosion.
- Computed tomography: To assess cholesteatoma formation. .

Management:

Aim:

- Closure of perforation of tympanic membrane.
- Restoration of hearing to the extent possible.
- Maintenance or restoration of normal anatomic configuration.

Medical management:

Aural toilet:

- Suction and cleaning
- Antibiotics (topical or systemic)

Treat with appropriate antibiotic based on antibiotic sensitivity pattern. The prevalence of MRSA, ESBL, Amp C and MBL producers are considered to be important.

Development of antimicrobial resistance is by far the most important one. New emerging strains develop resistance pattern to old classes of antibiotics.

Multidrug resistant organisms are resistant to three or more group of antibiotics with different mechanisms of action. Inadequate antibiotic therapy leads to emergence of multidrug resistant bacteria.

Surgical management:

closure of tympanic membrane perforation and correction of hearing loss needs appropriate surgical procedures like myringoplasty combined with cortical mastoidectomy.

Microbiology:

A wide range of organisms, both bacteria and fungi may be isolated, proportion of different organisms vary from study to study.

Bacteria are common cause of infection in CSOM patients in majority of the studies.

Commonest bacterial cause for ASOM are Streptococcus pneumoniae, Haemophilus influenza and Moraxella catarrhalis.[7]

The organisms that commonly play pathogenic role in CSOM are Pseudomonas aeruginosa and Staphylococcus aureus. Other bacterial agents are Proteus species, Klebsiella species, Escherichia coli, Enterobacter species and Citrobacter species.

The prevalence of Pseudomonas aeruginosa and Klebsiella in CSOM is estimated to be same i.e. 45.12% in a study by Rebecca John et al.[18]

The prevalence of Pseudomonas aeruginosa in CSOM is estimated to 43.2% followed by Staphylococcus aureus which is 31% in a study by Susmita Kumari sahu et al.[14]

In a study by Patigaroo et al[39], the most common organism cultured was Staphylococcus aureus (65%) followed by Pseudomonas aeruginosa (13%) and E coli (7%).

BACTERIAL CAUSES:

Pseudomonas aeruginosa:

Pseudomonas aeruginosa is ubiquitous, mostly saprophytic, being found in water, soil or other moist environment. Some of them are associated with human infection like CSOM, infected burns and hospital acquired infections. Pseudomonas aeruginosa which is notorious for drug resistance and established as a nosocomial pathogen. Drug resistance of Pseudomonas aeruginosa has been found to increase along with its frequency. High level resistance to

antimicrobials and it's ability to form biofilms complicate the therapeutic outcome.

Pseudomonas aeruginosa belongs to family Pseudomonadaceae (rRNA group Ⅰ) and genus Pseudomonas. In phenotypic classification of the Pseudomonads, it belongs to fluorescent group of rRNA group Ⅰ. Pseudomonas aeruginosa produces the distinctive blue, water soluble pigment pyocyanin.

Identification of Pseudomonas aeruginosa can be made whenever the following characters are observed.[3]

- Gram negative rod
- Catalase positive
- Oxidase positive
- Typical smell (fruity grape like odor of aminoacetophenone)
- Actively motile by a polar flagellum
- Colony morphology:

1. On blood agar appear as large colonies with metallic sheen, mucoid, rough or pigmented (pyocyanin) and often beta haemolytic.

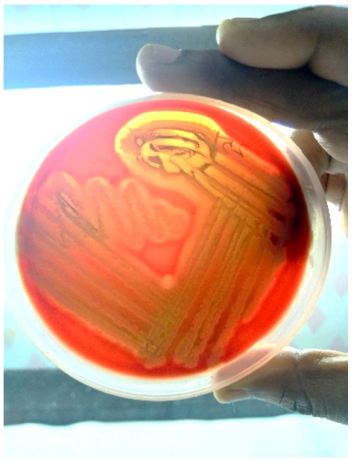

Fig 2: Beta haemolysis on Blood agar plate

2. On MacConkey agar, there are non lactose fermentingcolonies.

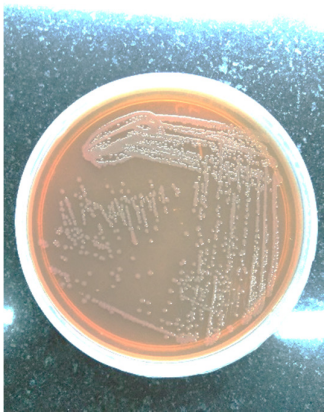

Fig 3: Non lactose fermenting colonies on Mac conkey agar plate

3. Pseudomonas aeruginosa can grow in Dettol or cetrimide selective medium. It is resistant to many disinfectants including quaternary ammonium compounds. One of the most satisfactory selective media is Pseudomonas

Isolation Agar which contains pigment enhancing components and the selective agent irgasan (2,4,4 trichloro 2 hydroxyphenyl ether)

Fig 4: Cetrimide agar – selective media.

Bio chemical reactions:

Fig 5: Bio chemical reactions

Catalase	Positive
Oxidase	Positive
Motility	Motile
Glucose	Fermenter
Lactose	Non fermenter
NO_3 to NO_2	Positive
NO_2 to N_2	Variable
Citrate	Variable
Urease	Variable
Lysine decarboxylase	Negative
Arginine dihydrolase	Positive
Ornithine decarboxylase	Negative

Fig 6: Sugar fermentation

Fig 7: LAO decarboxylase test.

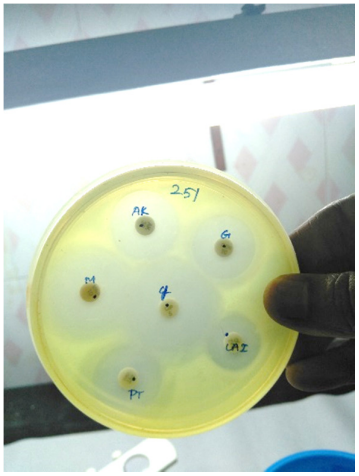

Fig 8: Antimicrobial susceptibility pattern

Performance of antimicrobial susceptibility test is carried with amikacin, gentamicin, ciprofloxacin, ceftazidime, meropenem and pip-taz as per CLSI 2019 guidelines. Pseudomonas aeruginosa may develop resistance during prolonged therapy with all antimicrobial agents. Therefore, isolates that are initially susceptible may become resistant within 3 to 4days after initiation of therapy.[61]

Virulence factors of P aeruginosa:

- ✓ Alginate – capsular polysaccharide
- ✓ Pili
- ✓ Neuraminidase
- ✓ Lipopolysaccharide
- ✓ Exotoxin A
- ✓ Enterotoxin
- ✓ Exoenzyme S
- ✓ Phospholipase C
- ✓ Elastase
- ✓ Leukocidin
- ✓ Pyocyanins

Staphylococcus aureus:

Staphylococcus aureus belongs to micrococcaceae family and genus staphylococcus. Staphylococci are non-motile, non-spore forming, catalase positive, gram positive cocci arranged in grape like clusters.

Some of the pathogenic staphylococci produce an enzyme called **coagulase**, and detection of this enzyme is used to identify these organisms.

Staphylococcus aureus is by far the most important human pathogen among the staphylococci. It is found in the external environment and in the anterior nares of 20-40% of adults. Other sites of colonization include

intertriginous skin folds, the perineum, the axillae and the vagina. It produces infections which range from localised pyogenic infections to life-threatening systemic infections.

Virulence factors:[3]

Cell wall associated factors – peptidoglycan, teichoic acid, cell surface adhesin (clumping factor) and protein A

Toxins:

- Membrane active toxins – haemolysins (alpha, beta, gamma, delta), leucocidin (Panton valentine toxin)
- Epidermolytic toxin (exfoliative toxin)
- Enterotoxin
- Toxic shock syndrome toxin

Extracellular enzymes: coagulase, heat stable thermonuclease, deoxyribonuclease, staphylokinase, hyaluronidase, lipase and protease.

Laboratory diagnosis:

Direct gram stain – GPC in clusters.

Culture – nutrient agar- golden yellow pigmented colonies.

Blood agar – colonies with narrow zone of haemolysis.

Selective media – mannitol salt agar, salt milk agar and Ludlam's medium

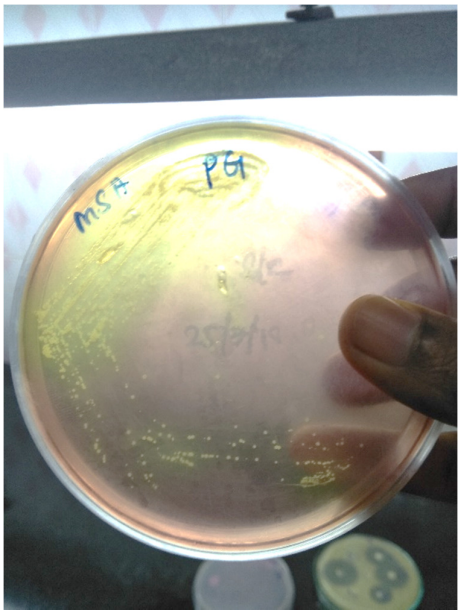

Fig 9: Mannitol salt agar - Selective media

Mannitol salt agar:

It is a selective and differential growth medium.

Selective medium: it contains a high concentration of salt, making it selective for gram positive bacteria.

Differential medium: if an organism can ferment mannitol, an acidic by-product is formed that cause the phenol red in the agar to turn yellow.

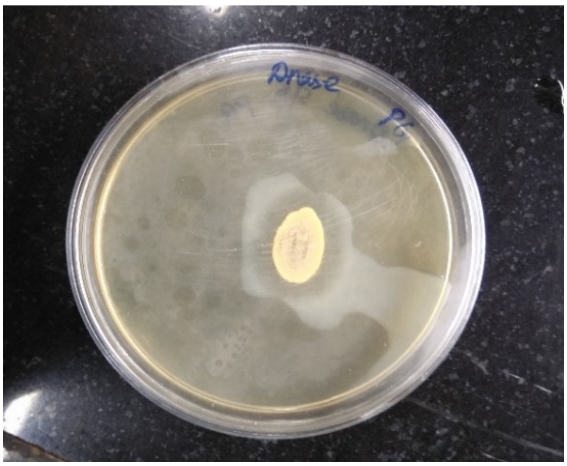

Fig 10: Deoxyribonuclease agar – clear halo around the colonies.

Principle:

To detect deoxyribonuclease activity of Staph aureus.

Positive DNase activity is visualised as clear zones around colonies when the plates are flooded with 1N hydrochloric acid.

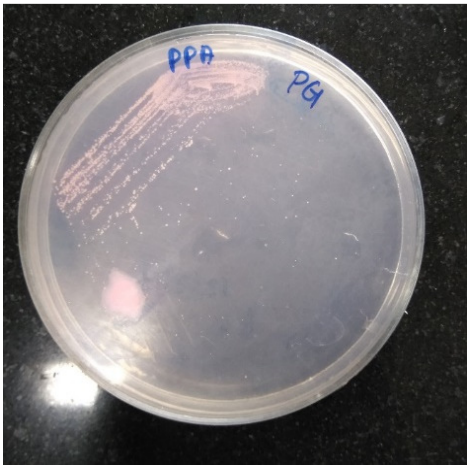

Fig 11: Phenolphthalein Phosphate agar – Identification of phosphatase positive Staph aureus.

Principle:

Phosphatase has been implicated as a virulence factor for Staph aureus. Phenolphthalein phosphate serves as a substrate for the phosphatase enzyme. When exposure to ammonia vapour, the liberated phenolphthalein gives a bright pink red colouration.

Catalase	**Positive**
Coagulase (slide and tube)	Positive
DNase test	Positive
Phosphatase test	Positive
Mannitol	Fermenter
Gelatin liquefaction	Positive
Urease	variable

Fungal causes:

Candida:

Candida is the commonest fungal agents infecting middle ear mucosa. It is yeast like fungi. It is found mainly as secondary infection in individuals with some underlying immunocompromised conditions. The commonest pathogenic species of this genus is Candida albicans.

Laboratory diagnosis:

- **Direct examination:**

1. KOH mount: Place the specimen on a clean glass slide. Add a drop of 10% KOH. Place the coverslip over the KOH drop. Gently press to get rid of any air bubbles. Slides are examined under low power(10x) and high power (40x) objective lens to view any yeast cell and pseudo hyphae.
2. Gram's staining: is performed to see presence of yeast and pseudo hyphae of candida species. The yeast cells are approximately 4-8µm with budding and pseudo hyphae. The gram's smear may be visible as gram negative also due to over decolourisation otherwise all the fungi are gram positive.

- **Fungal culture:**

The clinical specimens can be cultured on Sabouraud dextrose agar with antibacterial antibiotics and incubated at 25℃ and 37℃. The colonies appear in 2-3 days as cream coloured, smooth and pasty. The LCB mount is prepared from the colonies to examine for the presence of yeast cells and pseudo hyphae. Gram staining may also be performed from the culture isolates.

The growth of Candida species is also seen on Tetrazolium Reduction Medium (TRM) and compared to the standard colors to identify various species.

The Candida isolates are identified by standard protocols that include germ tube formation, chlamydospore production and sugar fermentation and sugar assimilation tests.

- **Germ tube test:**

This procedure is used for presumptive identification of candida species and is called germ tube test (GTT). The colony is inoculated with normal human serum and incubated at 37℃ for 2-4hrs. A drop of suspension is kept over the slide and coverslip is placed over the drop. The slide is examined under the low power (10x) and high power (40x) microscope. The germ tubes are seen as long tube-like projections extending from the yeast cells. There is no constriction at the point of attachment to the yeast cell as seen in case of pseudo hyphae. The demonstration of the germ tube is also known as **Reynolds-Braude phenomenon**.

Dalmau culture plate:[66]

The Dalmau plate culture on corn meal agar is commonly used to observe chlamydospore production in yeasts. Corn meal – Tween agar can also be prepared by adding Tween 80 (polysorbate). The role of Tween 80 is to decrease surface tension thereby stimulating production of chlamydospores.

Procedure:

- Take a cornmeal agar added with 1% Tween 80 in a 90mm plate.
- Load a sterile straight wire loop with the test organism.

- Make 2-3 streaks (approximately the streaks were 4cm long and 1cm apart) by cutting into the agar with the edge of sterilised loop.
- Then place the coverslip over the part of streaks. The streaks must be projected beyond the coverslip and provided anaerobic environment at the margins of the coverslip.
- Incubate at 22°c for 48hrs.
- Then the plate was examined under microscope with low and high power magnifications.

Interpretation:

Morphological features like true hyphae, pseudohyphae, terminal chlamydospores were seen.

Aspergillus species:

It is the commonest opportunistic fungal disease after candidiasis. The hyphae of genus aspergillus being hyaline in nature hence it is also a type of hyalohyphomycosis but is popularly known by its independent and well-established name, i.e. aspergillosis.

Aspergillus species are saprotrophic molds in nature and found in decaying organic matter worldwide. Three of them are consistently and regularly encountered as etiological agents of over 95% diseases caused by members of genus which are: Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger.

Laboratory diagnosis:

KOH mount: Characteristic narrow septate hyaline hyphae with acute angle branching.

Culture: Specimens are inoculated onto SDA and incubated at 25℃. Species identification is based on macroscopic and microscopic (LPCB mount) appearance of the colonies.

- Colonies consist of hyaline septate hyphae from which conidiophores arise which end at vesicles.
- From the vesicle, finger like projections of conidia producing cells arise called phialides or sterigmata. Phialides are arranged either in one or two rows, the first row is called metulae.

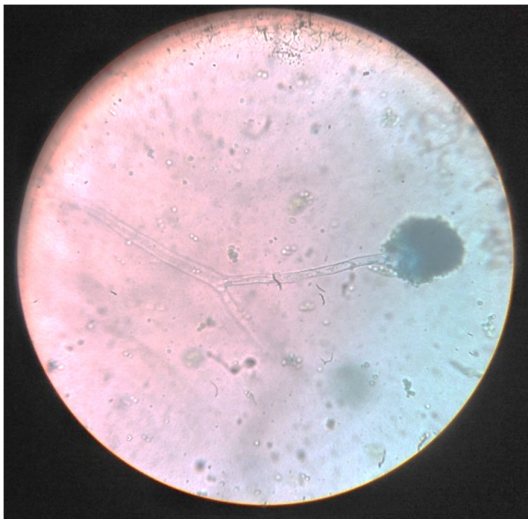

Fig 12: Aspergillus flavus in LPCB wet mount.

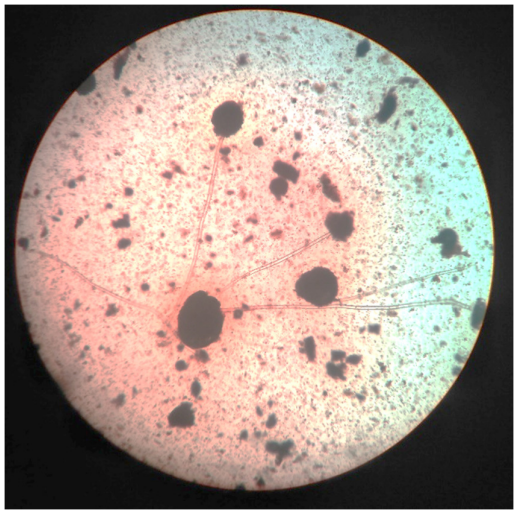

Fig 13: Aspergillus niger in LPCB wet mount.

Slide culture: [5]

Though this is a tedious procedure, it gives the most accurate in situ microscopic appearance of the fungal colony.

Procedure:

- A sterile slide is placed on a bent glass rod in a sterile Petri dish.
- Two square agar blocks measuring around $1cm^2$ (smaller than the coverslip) are placed on the slide.
- Bits of fungal colony are inoculated onto the margins of the agar block.
- Cover-slip is placed on the agar block
- Petri dish is incubated at 25°c.
- LPCB mount are made both from the cover-slip and the underneath slide.

Interpretation:

The final interpretation of the fungal type can be made with the type of hyphae arrangement of conidiophores, staining character etc….

Interpretation of fungal cultures:

Inoculated SDA slants were incubated at room temperature for minimum of 4weeks before discard it as negative. These inoculated slants were inspected daily during the first week and twice weekly during the next three weeks for growth.

Identification of yeast was done by gram staining, germ tube test, morphology on corn meal agar and biochemical tests recommended by CLSI. In case of filamentous fungi, identification was done by LPCB mount to study the morphology of hyphae and conidial arrangement.

CLASSIFICATION OF ANTIBIOTICS[5]

I.CELL WALL SYNTHESIS INHIBITORS

Beta lactam group of antibiotics :

Penicilin

- Penicilin G,Penicillin V, Benzathine Penicillin,Procaine Penicillin
- Penicillinase resistant penicillin - Methicillin, Cloxacillin ,Oxacillin, Dicloxacillin
- AminoPenicillin - Ampicillin,Amoxycillin
- AntiPseudomonal penicillin - Piperacillin,Ticarcillin,Carbenicillin

Cephalosporins

- 1st Generation - Cefazolin,Cephalexin,Cefuroxime
- 2nd Generation - Cefoxitin,Cefotetan,Cefaclor
- 3rd Generation - Cefotaxime,Ceftriaxone,Ceftazidime
- 4th Generation - Cefepime,Cefpirome
- 5th Generation - Ceftabiprole,Ceftaroline

Carbapenams

- Ertapenam,Meropenam,Imipenam,Doripenam

Aztreonam

Glycopeptides

- Vancomycin,Daptomycin

Fosfomycin

Bacitracin

II.PROTEIN SYNTHESIS INHIBITORS

Acts at 30S Ribosomal subunit

- Aminoglycosides - Amikacin,Gentamycin,
- Tetracyclines - Doxycycline,Minocycline

Acts at 50s Ribosomal subunit

- Macrolides - Erythromycin, azithromycin Clarithromycin, Rozithromycin
- Lincosamides - Clindamycin
- Chloramphenicol
- Streptogramins - Quinopristin,Dalfopristin
- Linezolid
- Isoleucine t-rna synthetase Inhibitor - Mupirocin

III.NUCLEIC ACID SYNTHESIS INHIBITORS

DNA SYNTHESIS INHIBITORS

- Flouroquinolones
- 1st Generation Norfloxacin,Ofloxacin,Ciprofloxacin
- 2nd Generation Levofloxacin,Moxifloxacin,Sparfloxacin,
- Metronidazole,Ornidazole,Tinidazole, Naidixic acid

RNA SYNTHESIS INHIBITOR : Rifampin

IV.MYCOLIC ACID SYNTHESIS INHIBITOR : Isoniazid

V.FOLIC ACID SYNTHESIS INHIBITOR : Trimethoprim and sulfonamides

VI. ANTIBIOTICS ACTING ON CELL MEMBRANE

Gramicidin – Forms pores

Daptomycin – Forms channels

Polymixins- 5 Types (A-E)

INTRINSIC RESISTANCE[5]

Gram positive bacteria - Aztreonam

Gram negative bacteria - Vancomycin

Klebsiella - Ampicillin

Anaerobes - Aminoglycosides

Pseudomonas - Cotrimoxazole,Tetracyclines and Chloramphenicol

Enterococcus - Aminoglycosides, cephalosporins

Aerobes - Metronidazole

Stenotropomonas - Carbapenams

MECHANISM OF RESISTANCE TO ANTIMICROBIAL AGENTS:

Enzymatic inactivation

Beta lactams - Beta lactamase, penicillinase

aminoglycosides - Aminoglycoside modifying enzymes

Altered receptors

Beta lactams - Altered PBPs (pencillin binding proteins)

Ribosomal alterations - Tetracycline, erythromycin

DNA gyrase alterations - Fluoroquinolones

Altered bacterial enzymes - Trimethoprim, Sulfamethoxazole

Altered antibiotic transport

Porins, Active transport from bacterial cells, decreased efflux - Gram negative bacteria ,

Antimicrobial resistance:

Emergence of antibacterial resistance and production of extended spectrum beta lactamase (ESBLs) are responsible for the frequently observed empirical therapy failures. There is rapid dissemination of ESBLs producing Enterobacteriaceae isolates, particularly Escherichia coli and Klebsiella pneumoniae.

Screening test for ESBL production:

Detection of non-susceptibility of the organisms to indicator oxyiminocephalosporins namely ceftriaxone, ceftazidime, cefotaxime, cefpodoxime and/or aztreonam identifies ESBL production.

Phenotypic confirmatory test for ESBL production:

Screening test positive organisms are subjected to the confirmatory test. This test is done using both cefotaxime and ceftazidime alone and in combination with clavulanic acid.

Procedure: 0.5McFarland suspension of the test organism in normal saline prepared from 18 to 24-hour subculture is streaked on to MHA plate and the disks are placed. The plates are incubated overnight at 37℃ and the zone sizes are measured next day.

Interpretation: ≥ 5mm increase in zone diameter for either antimicrobial agent when tested in combination with clavulanic acid than its zone when tested alone.

MBL producing NFGNB:

- MBL are a diverse set of enzymes that catalyses the hydrolysis of a broad range of beta lactam drugs including carbapenems.
- Class B metallo beta lactamases have a broad substrate spectrum and can catalyse the hydrolysis of virtually all the beta lactam antibiotics with the exception of monobactams. They are not inhibited by inhibitors such as clavulanate, sulbactam/tazobactam that are effective against serine based, class A beta lactamases.
- MBLs are now considered as a serious problem in antibiotic therapy because of the spread of IMP and VIM type Metallobetalactamase in

gram negative pathogens including Enterobacteriaceae, Pseudomonas aeruginosa and Acinetobacter baumannii

- These isolates are confirmed by Imipenem EDTA combined disk test.

Imipenem EDTA combined disk test

Prepare 0.5McFarland bacterial suspension from an overnight growth on BAP.

Inoculate surface of MHA plate using this suspension as per standard disk diffusion method

Place a 10µg imipenem disk at a distance of 10mm from 10µg /750µg imipenem-EDTA disk

Incubate at 37°c

Interpretation:

Disk containing imipenem-EDTA showing inhibition zone of > 8-15mm than the imipenem disk is considered to be a MBL producer.

Carbapenemase producing NFGNB

- Carbapenemases are beta-lactamases that hydrolyse pencillin, in most cases cephalosporins and to various degrees of carbapenems and monobactams.
- Most commonly observed in Pseudomonas aeruginosa and Klebsiella pneumoniae.
- Carbapenemases are enzymes, encoded by genes on transposable elements located on plasmids.

- Carba NP test (conventional and commercial) for suspected carbapenemase production in Pseudomonas and Acinetobacter species.

Modified carbapenem inactivation methods for suspected carbapenemase production in Pseudomonas aeruginosa

mCIM is not routinely done as a diagnostic method in microbiology laboratory, done only for epidemiological or infection control purposes.[60]

Procedure:

- Emulsify a 10µl loopful of the test organism (P aeruginosa) from an overnight blood agar plate in 2ml TSB.
- Vortex for 10-15 seconds.
- Add a 10µg meropenem disk to each tube using sterile forceps.
- Incubate at 35°c ± 2°c in ambient air for 4hrs ± 15min.
- Just before or immediately following completion of the TSB – meropenem disk suspension incubation, prepare a 0.5 McFarland suspension of ATCC E coli 25922 in nutrient broth or saline.
- Inoculate an MHA plate with ATCC E coli 25922 suspension as for the routine disk diffusion procedure making sure the inoculum suspension preparation and MHA plate inoculation steps are each completed within 15min. Allow the plates to dry for 3-10min before adding the meropenem disks.

- Remove the meropenem disk from each TSB-meropenem disk suspension using a 10µl loop by placing the flats side of the loop against the flat edge of the disk and using surface tension to pull the disk out of the liquid. Carefully drag and press the loop along the inside edges of the tube to expel excess liquid from the disk. Continue using the loop to remove the disk from the tube and then place it on the MHA plate previously inoculated with the meropenem susceptible ATCC E coli 25922.
- Invert and incubate the MHA plates at 35°c ± 2°c in ambient air for 18 - 24hrs.
- Following incubation, measure the zones of inhibition as for the routine disk diffusion method.

Interpretation:

Carbapenemase positive:

Zone diameter of 6-15mm or presence of pinpoint colonies within a 16-18mm zone. If the test isolate produces a carbapenemase, the meropenem in the disk will be hydrolysed and there will be no inhibition or limited growth inhibition of the meropenem susceptible ATCC E coli 25922.

Carbapenemase negative:

Zone diameter of ≥ 19mm. If the test isolate does not produce carbapenemases, the meropenem in the disk will not be hydrolysed and will inhibit growth of the meropenem susceptible ATCC E coli 25922.

Carbapenemase indeterminate:

- Zone diameter of 16-18mm
- Zone diameter of ≥19mm and the presence of pinpoint colonies within the zone.
- The presence or absence of a carbapenemase cannot be confirmed.

MRSA detection methods:

- Disc diffusion method – by cefoxitin/oxacillin discs.
- MIC detection – dilution methods (agar/broth), E-strip.
- Detection of mec A gene by molecular method.

Cefoxitin disk diffusion method:

Resistance to staphylococcus to penicillinase resistant pencillins is due to the presence of an altered pencillin binding protein (PBP2a) in the cell wall coded by the gene mecA. PBP2a has low affinity for binding all beta lactam drugs. Therefore, S aureus possessing mecA is resistant to pencillins and penicillinase resistant pencillins like methicillin, oxacillin, cloxacillin and referred as Methicillin Resistant Staphylococcus aureus. Cefoxitin is used as a surrogate marker for detection of mecA mediated oxacillin resistance.

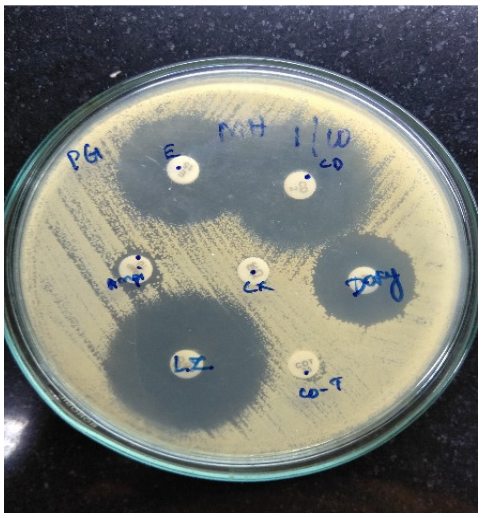

Fig 14: Cefoxitin disc diffusion method

Procedure:

Prepare a lawn culture of S aureus on MHA plate aseptically and cefoxitin disk (30µg) is placed on the inoculated agar surface. Invert plate and incubate at 33-35°c for 16-18hrs.

Interpretation:

S aureus and S lugdunensis showing diameter of zone of inhibition ≤ 21mm is considered as mecA mediated oxacillin resistant.

For CONS zone diameter ≤ 24mm is considered as mecA mediated oxacillin resistant.

Oxacillin resistant S aureus isolates should be reported as resistant to all beta lactam agents except those with anti MRSA activity like ceftaroline and ceftobiprole.

Inducible clindamycin resistance in S aureus detection method:

Mechanism of action of resistance

Erythromycin (macrolide) and clindamycin (lincosamide) represent two distinct classes of antimicrobial agents that inhibit protein synthesis by binding to the 50S ribosomal subunits of bacterial cells. In Staphylococci, resistance to both of these antimicrobial agents can occur through methylation of their ribosomal target site. Such resistance is typically mediated by **erm** genes.

Clinical significance of inducible clindamycin resistance:

Macrolide lincosamide streptogramin B (MLS B) resistance which is mediated by target site modification mechanism, results in resistance to erythromycin, clindamycin and streptogramin B. This mechanism can be,

- Constitutive, where the rRNA methylase is always produced, if in vitro testing is done, S aureus isolates with constitutive resistance are resistant to Erythromycin and Clindamycin.
- Or can be inducible, where methylase is produced only in the presence of inducing agent. Isolates with inducible resistance are resistant to erythromycin but appear susceptible to clindamycin in routine in-vitro testing.
- Inducible clindamycin resistance in staphylococci can be detected by disk diffusion method using clindamycin and erythromycin disks. “D”

shaped zone of inhibition around clindamycin disk proximal to erythromycin disk indicates erythromycin has induced clindamycin resistance.

Procedure:

A lawn culture of the test organism is made on Muller Hinton Agar plate. Erythromycin (15µg) and clindamycin (2µg) disks are placed 15-26mm apart. Incubate the plate under 35°c ± 2°c for 16-18hrs.

Interpretation:

Flattening of the zone of inhibition adjacent to the erythromycin disk – positive

Hazy growth within the zone of inhibition around clindamycin, even if no D zone apparent – positive.

MATERIALS AND METHODS

This prospective study was conducted at the department of microbiology, Thanjavur Medical College in association with Department of Ear, Nose and Throat.

STUDY PERIOD

April 2018 – June 2019

STUDY POPULATION

The study population consisted of 100 patients, including all age groups attending otolaryngology opd (outpatient department) with signs and symptoms of CSOM.

ETHICAL CLEARANCE

Institutional Ethical Committee approval was obtained, before the commencement of the study.

From the study group, informed consent was obtained by asking structural questionnaire, the patients were interviewed.

INCLUSION CRITERIA

- Patients who had active ear discharge for atleast three months
- Clinically diagnosed CSOM patients with ear discharge, who did not receive antimicrobial therapy for the last 7 days

- Patients of any age, both gender, discharge from unilateral or bilateral ears were included.

EXCLUSION CRITERIA

- Patients with history of using antibiotic either systemic or local in the form of ear drops for last 7 days.
- Patients with ear discharge of less than 3 months duration
- Patients with ear discharge with intact tympanic membrane (otitis externa)
- Congenital ear or hearing problems
- Obstructed middle ear (e.g. polyp)
- Patients with ear discharge due to cholesteatoma.
- Malignancy
- Previous ear surgery

DATA COLLECTION:

Complete data about the patient's name, age, sex, hospital number, date of collection of pus sample, history of presenting illness were collected from the patients. Past history of any upper respiratory tract infection, acute otitis media were also collected from the patient.

SAMPLE COLLECTION:

Specimen: Pus (ear discharge)

Under aseptic precautions, the external ear canal was cleaned by wiping with sterile cotton with 70% alcohol and allowed to dry. Under illumination using a sterile auditory speculum, the sterile swab stick was gently introduced, rotated and removed with precaution and immediately put into its container so as not to touch the external ear canal or any part of the skin. Sterile saline 0.5-1ml may be added to prevent the swab from drying. Three swabs from a single ear were collected in this manner, labelled and processed as soon as possible in our microbiology laboratory.[15]

Direct microscopy (first swab)

The first swab was used to make a smear clean grease free glass slide for gram stain examination and direct microscopy of specimen in KOH for fungal examination.

- **Direct gram stain:**

  The report had to include the following information.

  - Presence and number of pus cells.
  - Presence of epithelial cells.
  - Gram reaction of the bacteria, whether gram positive or gram negative.
  - Morphology of the bacteria, whether cocci, bacilli or coccobacilli.
  - Presence of yeast cells.

- **10% KOH (potassium hydroxide) mount:**

  To detect the presence of fungal elements.

Bacterial culture plates (second swab):

The second swab was used for the bacterial culture and then plated into the nutrient agar, mac conkey agar and 5% sheep blood agar. All culture plates were incubated at 37℃ under aerobic condition. Plates were evaluated for growth at 24hrs and isolates were identified by using colony morphology, microscopy and standard biochemical tests. Antimicrobial susceptibility testing of bacterial isolates was done by Kirby-Bauer disk diffusion method according to the CLSI guidelines.

Fungal culture (third swab)

The third swab was inoculated into the Sabouraud's dextrose agar and was kept up to 4weeks.

Gram stain:

This staining technique was originally developed by Hans Christian Gram (1884).

Procedure:[5]

- The smear made on a slide from specimen. It is dried and then heat fixed.
- Primary stain: the smear is stained with methyl violet for one minute. Then the slide is rinsed with water

- Mordant: gram's iodine is poured over the slide for one minute. Then the slide is rinsed with water.
- Decolorization: pouring of few drops of decolorizer e.g. acetone (for 1-2 sec). slide is immediately rinsed with water. Decolorizer removes the primary stain from gram negative bacteria while the gram-positive bacteria retain the primary stain.
- Counterstain: secondary stain such as diluted carbol fuchsin is added for 30 seconds. It imparts pink or red colour to the gram-negative bacteria.

Colony morphology:

The appearance of bacterial colony helps in preliminary identification.

- Size: in millimetres. E.g. pin head size is characteristic of staphylococcal colony.
- Shape: circular or irregular.
- Surface: glistening or dull.
- Edge: entire, crenated, lobate, undulated or filamentous.
- Elevation: flat, raised, convex, umbonate or pulvinate.
- Consistency: mucoid, friable, firm or butyrous
- Density: opaque, translucent or transparent
- Haemolysis on blood agar
- Color of the colony: colonies may be colored due to pigment production.

- Diffusible pigment: they diffuse throughout the media.
- Non diffusible pigment: colonies are colored, not the surrounding media.

MacConkey agar:[1]

Peptone	-	20g
Sodium taurocholate	-	5g
Water	-	1lit
Agar	-	20g
Neutral red solution, 2% in 50% ethanol	-	3.5ml
Lactose, 10% aqueous solution	-	100ml

Dissolve the peptone and taurocholate (bile salt) in the water by heating. Add the agar and dissolve it in the steamer or autoclave. If necessary, clear by filtration. Adjust the pH to 7.5. Add the lactose and the neutral red, which should be well shaken before use and mix. Heat in the autoclave with 'free steam' 100℃ for 1hr, then at 115℃ for 15min. Pour plates. The medium should be a distinct reddish-brown colour.

Tryptose blood agar:

Tryptose – 10g

Beef extract – 3g

Yeast extract – 3g

Sodium chloride – 5g

Agar – 15g

Distilled water – 1lit

Ph – 7.1

The medium was sterilised and allowed to cool to 45℃ and aseptically added 5% defibrinated sheep blood. Mixed thoroughly and poured into sterile petri plates and stored in refrigerator.

Biochemical reactions:

Indole test:[2]

Medium:

- Peptone (brand containing sufficient tryptophane) – 20g
- Sodium chloride, Nacl – 5g
- Distilled water – 1lit

Adjust the pH to 7.4 dispense and sterilise by autoclaving at 121℃ for 15min.

Kovac's reagent:

- Amyl or isoamyl alcohol – 150ml
- Para dimethyl amino benzaldehyde – 10g
- Concentrated hydrochloric acid – 50ml

Method:

Inoculate medium and incubate for 48hr at 37℃. add 0.5ml kovac's reagent and shake gently. A red colour in the alcohol layer indicates positive reaction.

Methyl red test:

Medium (glucose phosphate peptone water)

- Peptone – 5g
- Dipotassium hydrogen phosphate – 5g
- Water – 1lit
- Glucose, 10% solution – 50ml

Methyl red indicator solution

- Methyl red – 0.1g
- Ethanol – 300ml
- Distilled water – 200ml

Method:

Inoculate the liquid medium from a young agar slope culture and incubate at 37°c for 48hrs. Add about five drops of methyl red reagent. Mix and read immediately. Positive tests are bright red and negative are yellow.

Voges-Proskauer (acetoin production) test:

Medium:

glucose phosphate peptone water.

Method:

Incubate at 37°c for 48hrs. Add 1ml of 40% potassium hydroxide and 3ml of a 5% solution of alpha naphthol in absolute ethanol. A positive reaction is indicated by the development of a pink colour in 2-5min, becoming crimson in 30min. The tube can be shaken at intervals to ensure maximum aeration.

Citrate utilisation test:

Medium (Simmons' citrate)

Ammonium dihydrogen phosphate – 1g

Potassium dihydrogen phosphate – 1g

Sodium chloride -5g

Sodium citrate -2g

Magnesium sulphate – 0.2g

Agar - 15g

Bromothymol blue – 0.8g

Distilled water – 1lit

pH – 6.9

Method:

Inoculate from a saline suspension of the organism to be tested. Incubate for 96hr at 37°c.

Positive – blue colour and streak of growth

Negative – original green colour and no growth.

Urease test:

Christensen's medium

Peptone – 1g

Sodium chloride – 5g

Dipotassium hydrogen phosphate – 2g

Phenol red – 6ml

Agar – 20g

Distilled water – 1lit

Glucose, 10% solution – 10ml

Urea, 20% solution – 100ml

Method:

Inoculate heavily over the entire slope surface and incubate at 37°c. Examine after 4hr and overnight incubation, no tube being reported negative until after 4days' incubation. Urease positive cultures change the colour of the indicator to purple – pink.

Catalase test:

Reagent – 3% hydrogen peroxide

Method:

Culture to be tested is picked from a nutrient agar slope with a clean thin glass rod and this is inserted into hydrogen peroxide solution held in a small, clean tube. The production of gas bubbles indicates a positive reaction.

Oxidase test:

Wet filter paper method:

A strip of filter paper is soaked with a freshly made 1% solution of the tetramethyl Para phenylene diamine dihydrochloride reagent and then at once used by rubbing a speck of culture on it with a platinum loop. A positive reaction is indicated by an intense deep purple hue, appearing within 5-10sec and a negative reaction by absence of coloration.

Triple sugar iron agar:

Medium:

Beef extract – 3g

Yeast extract – 3g

Peptone – 20g

Glucose – 1g

Lactose – 10g

Sucrose – 10g

Ferric citrate – 0.3g

Sodium chloride – 5g

Sodium thiosulphate – 0.3g

Agar – 12g

Phenol red, 0.2% solution – 12ml

Distilled water – 1lit.

Method:

Streak a heavy inoculum over the surface of the slope and stab into the butt. Incubate aerobically at 37°c for 24hrs.

Interpretation:

Slant/butt	Colour	Utilisation
Alkaline/acid	Red/yellow	Glucose only fermented; Peptones utilised.
Acid/acid	Yellow/yellow	Glucose fermented; lactose and/or sucrose fermented
Alkaline/alkaline	Red/red	No fermentation of glucose, lactose or sucrose.

Coagulase test:

Method:

Emulsify a colony to be tested in a tube of the 0.5ml plasma. Incubate at 35℃ for 4hrs. read as positive if any degree of clot formation. Read as negative tubes in which plasma remains wholly liquid.

Detection of motility by microscopy:

Hanging drop preparation is one of the easiest methods to observe motility in our clinical microbiology laboratory.

Procedure:

- Use a 'hollow ground' slide, i.e. a glass slide with a shallow, circular concavity in its centre.
- Take a clean coverslip; apply paraffin to the four corners of coverslip.
- Place a small drop of the liquid culture or suspension on the coverslip. Make sure that the suspension of culture is not so dense that the crowded organisms obscure one another. If necessary, dilute the suspension.
- Invert the slide over the coverslip, allowing it to adhere to the jelly. Then quickly turn around the slide so that the coverslip is uppermost. The drop will then be hanging from the coverslip in the centre of the concavity.
- Proceed to examine first with a low power objective(10x) and then with a high power one (40x)

Processing of fungus:

Fungus are much less abundant than bacteria in affected clinical material. Hence all mycological investigations like collection of specimen, microscopy and culture are crucial.

Gram stain:

This is the most important staining method in bacteriology as well as in mycology.

Gram positive oval budding yeast cells with pseudo hyphae were seen and identified as candida species.

Potassium hydroxide wet mount preparation:

Fungal elements can be detected by direct microscopic examination of material. Caution should be maintained while interpretation of hyphae, which may be confused with collagen fibre, cotton fibre or hair etc.

Reagent:

Potassium hydroxide - 10g

Glycerol - 10ml

Distilled water - 80ml

Procedure:

- Take a clean grease free slide.
- Place a drop of 10% KOH on a slide and emulsify the specimen with it by using a loop.
- Cover the drop with a coverslip gently.
- Leave it for 5-10min.
- Examine the slide under low power(10x) and high power (40x) magnifications.

- Examine the slide for demonstration of fungal elements like budding yeast cells, septate or aseptate hyphae etc.

Sabouraud's dextrose agar:

It is the most commonly used media in diagnostic mycology. Fungal culture by using SDA is frequently performed for isolation and correct identification of fungi.

Components:

- Peptone – 10g
- Dextrose – 40g
- Agar – 20-30g
- Distilled water – 100ml
- pH – 5.4

Germ tube test:

It is a test to differentiate Candida albicans from non albicans. It is also called Reynolds Braude phenomenon.

Procedure:

- Lightly touch a single colony with a loop or Pasteur pipette
- Emulsify the colony in 0.5ml of horse or other serum in a small test tube.

- Incubate at 37℃ for 2-4hrs. Prolonged incubation is not recommended as mycelium production can obscure the germ tubes.
- Place one drop of suspension on the slide and place the coverslip over the drop.
- Examine the slide under low power(10x) and high power(40x) magnifications.

Interpretation:

Observe any cell showing production of germ tube (long tube-like projections extending from yeast cells) under the microscope.

Lactophenol cotton blue staining

It is used to study the microscopic appearance of the fungal isolate grown in culture.

Components:

- Phenol crystals – 20g (phenol act as disinfectant)
- Lactic acid - 20ml (preserves the morphology of fungi)
- Glycerol – 40ml (prevents drying)
- Distilled water – 20ml
- Cotton blue (or methyl blue) – 0.075g (stains the fungal elements blue)

Procedure:

- Place a drop of LPCB on a clean grease free glass slide.

- Remove a small portion of colony and the supporting agar at a point between the centre and periphery and place it in a drop of LPCB.
- Gently tease out a fragment of the culture with needles or straight wires.
- Apply a cover-slip, avoiding bubbles, and exert gentle pressure if the fungus fragments do not lie flat.
- Remove any excess stain round the cover-slip with the edge of a piece of blotting paper.
- Let the stain penetrate. It may be satisfactory within a few minutes.
- Examine the slide under low power (10x) and high power (40x) magnifications.

Interpretation:

The fungal elements (hyphae and spores) can be observed.

Antimicrobial sensitivity pattern:

It was done by Kirby-Bauer disk diffusion method on Muller Hinton agar using appropriate antimicrobial drugs as per CLSI 2019 guidelines.

Muller Hinton agar is considered as the best medium to use for routine susceptibility testing of non-fastidious bacteria for the following reasons.

- It shows acceptable batch to batch reproducibility for susceptibility testing.
- It supports satisfactory growth of most non-fastidious pathogens.

Procedure:

- Isolated pure colonies of the test organism were inoculated in a peptone water broth and incubated at 35-37°c for 4-6hrs.
- The density of the organisms in broth was adjusted to approximately 1.5 x 10*8cfu/ml by comparing its turbidity with that of 0.5McFarland opacity standard tube.
- Lawn culture: the broth was inoculated on the medium by spreading with sterile swab.
- The plate was allowed to dry for 3-5min.
- Then antibiotic disks were applied by using sterile needle or forceps.
- The plates were incubated at 37°c overnight.
- Zone of inhibition was measured by using zone diameter measurement scale.

The following American type culture collection strains were used as quality control strains.

- Escherichia coli – ATCC 25922
- Staphylococcus aureus – ATCC 25923
- Klebsiella pneumoniae – ATCC 700603
- Pseudomonas aeruginosa – ATCC 27853

The antibiotic panel included in the antimicrobial sensitivity testing (CLSI guidelines)

Interpretative zone diameters for non-fermenters gram negative bacilli.

Antibiotic	Disk content in µg	Diameter of zone inhibition in mm Breakpoint		
		Sensitive Zone	Intermediate Zone	Resistant Zone
Amikacin	30µg	≥17	15-16	≤14
Gentamicin	10µg	≥15	13-14	≤12
Ceftazidime	30µg	≥18	15-17	≤14
Ciprofloxacin	5µg	≥25	19-24	≤18
Meropenem	10µg	≥19	16-18	≤15
Imipenem	10µg	≥19	16-18	≤15
Piperacillin/tazobactam	100/10µg	≥21	15-20	≤14

Interpretative zone diameter for Gram positive cocci

Antibiotic	Disk content in µg	Diameter of zone of inhibition in mm Breakpoint		
		Sensitive	Intermediate	Resistant
Pencillin G	10U	≥29	-	≤28
Cefoxitin	30µg	≥22	-	≤21
Erythromycin	15µg	≥23	14-22	≤13
Clindamycin	2µg	≥21	15-20	≤14
Cotrimoxazole	1.25µg/23.75µg	≥16	11-15	≤10
Ciprofloxacin	5µg	≥21	16-20	≤15
Linezolid	30µg	≥21	-	≤20
Gentamicin	10µg	≥15	13-14	≤12

Detection of MRSA by disk diffusion method:

Morphological similar colonies isolated from agar culture plate were inoculated into broth to reach 0.5 McFarland opacity standard. A lawn culture was made on the MHA plate and 30µg cefoxitin disk was placed on the surface of the MHA plate and incubated at 35℃ for 16-18hrs.

Interpretation:

Zone of inhibition ≤21mm – mecA positive.

Zone of inhibition ≥ 22mm – mecA negative.

Screening test for ESBL production:

Detection of non-susceptibility of the organisms to indicator oxyiminocephalosporins namely ceftriaxone, ceftazidime, cefotaxime, cefpodoxime and/or aztreonam identifies ESBL production.

Phenotypic confirmatory test for ESBL production:

Screening test positive organisms are subjected to the confirmatory test. This test is done using both cefotaxime and ceftazidime alone and in combination with clavulanic acid.

Procedure: 0.5McFarland suspension of the test organism in normal saline prepared from 18 to 24-hour subculture is streaked on to MHA plate and the disks are placed. The plates are incubated overnight at 37℃ and the zone sizes are measured next day.

Interpretation:

≥ 5mm increase in zone diameter for either antimicrobial agent when tested in combination with clavulanic acid than its zone when tested alone.

Molecular identification of Antimicrobial Resistant Gene

Materials & Methods: Pure Fast Bacterial DNA minispin purification kit [Lysozyme, Lysozyme digestion buffer, Proteinase-K, Binding buffer, Wash Buffer 1, Wash Buffer 2, Spin columns with collection tube and elution buffer. HELINI 2X Red dye PCR Master Mix, Agarose gel electrophoresis Consumables mecA Primer.

2X Master Mix:

It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and Red Dye PCR additives.

Agarose gel electrophoresis:

Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide is from HELINI Biomolecules, Chennai.

PCR:

HELINI Ready to use mecA gene Primer mix -

5µl/reaction PCR Product: 200bp

Bacterial DNA Purification

1. 1ml of overnight culture centrifuged at 6000rpm for 5min
2. Supernatant discarded

3. Pellet is suspended in 0.2ml PBS.
4. 180µl of lysozyme digestion buffer and 20µl of lysozyme [10mg/ml] added.
5. Incubated at 37C for 15min.
6. 400µl of Binding buffer, 5µl of internal control template and 20µl of proteinase K added, mixed well by inverting several times.
7. Incubated at 56ºC for 15min.
8. 300µl of Ethanol is added and mixed well.
9. The entire sample is transferred into the Pure Fast spin column. Centrifuged for 1 min. Discard the flow-through and place the column back into the same collection tube.
10. 500µl Wash buffer-1is added to the Pure Fast spin column. Centrifuged for 30-60 seconds and the flow-through is discarded. Place the column back into the same collection tube.
11. 500µl Wash buffer-2 is added to the Pure Fast spin column. Centrifuge for 30-60 seconds and the flow-through is discarded. Place the column back into the same collection tube.
12. Discard the flow-through and centrifuge for an additional 1 min. This step is essential to avoid residual ethanol.
13. Pure Fast spin column is transferred into a fresh 1.5 ml micro centrifuge tube.

14. 100µl of Elution Buffer is added to the centre of Pure Fast spin column membrane.
15. Incubate for 1 min at room temperature and centrifuge for 2 min.
16. Discard the column and store the purified DNA at -20°C. Quality and Quantity of extracted DNA is checked by loading in 1% agarose gel and 5µl of extracted DNA is used for PCR amplification.

PCR Procedure:

1. Reactions are set up as follows;

   Components Quantity

   HELINI Red Dye PCR Master mix - 10µl

   HELINI Ready to use - Primer Mix - 5µl

   Purified Bacterial DNA - 5µl

   Total volume - 20µl

2. Mixed gently and spinned down briefly.
3. It is placed into PCR machine and it was programmed as follows:

   Initial Denaturation at 95ºC for 5 minutes

   Denaturation at 94ºC for 30seconds

   Annealing at 58ºC for 30seconds for 35 cycles

   Extension at 72ºC for 30seconds

   Final extension at 72º C for 5 minutes

   1. 2% agarose gel is prepared.

Electrophoresis is run at 50V till the dye reaches three fourth distances and the bands are observed in UV Transilluminator

Agarose gel electrophoresis:

1. 2% agarose gel is loaded
2. When the agarose gel temperature is around 60ºC, 5µl of Ethidium bromide is added
3. warm agarose solution is poured slowly into the gel platform.
4. The gel set is kept undisturbed till the agarose solidifies.
5. 1XTAE is poured buffer into submarine gel tank.
6. The gel platform is carefully poured into the tank. The tank buffer level is maintained 0.5cm above than the gel.
7. PCR Samples are loaded after mixed with gel loading dye along with 10µl HELINI 100bp DNA Ladder.
8. Electrophoresis is run at 50V till the dye reaches three fourth distance of the gel. Gel viewed in UV Transilluminator and observed the bands pattern

RESULTS

Statistical Analysis

Data were entered in the excel spread sheet and variables were coded accordingly. The statistical analyses were performed using Graph pad Prism version 5 software. Data were presented as frequency with proportion n(%) for categorical. Fisher's exact test (for sample <30) was used to compare the proportions between the groups as appropriate. $p<0.05$ was considered statistically significant.

Table 1. Frequency distribution of age category in the study.

S.No	Age category	Frequency (n)	Proportion (%)
1	< 1 year	1	1
2	>1 to 12 years	21	21
3	13 to 18 years	14	14
4	19 to 30 years	19	19
5	31 to 60 years	37	37
6	>60 years	8	8

Data are expressed as n and %. The total N=100.

Males and females within the age of 31-60 years suffered more from CSOM when compared to other age groups.

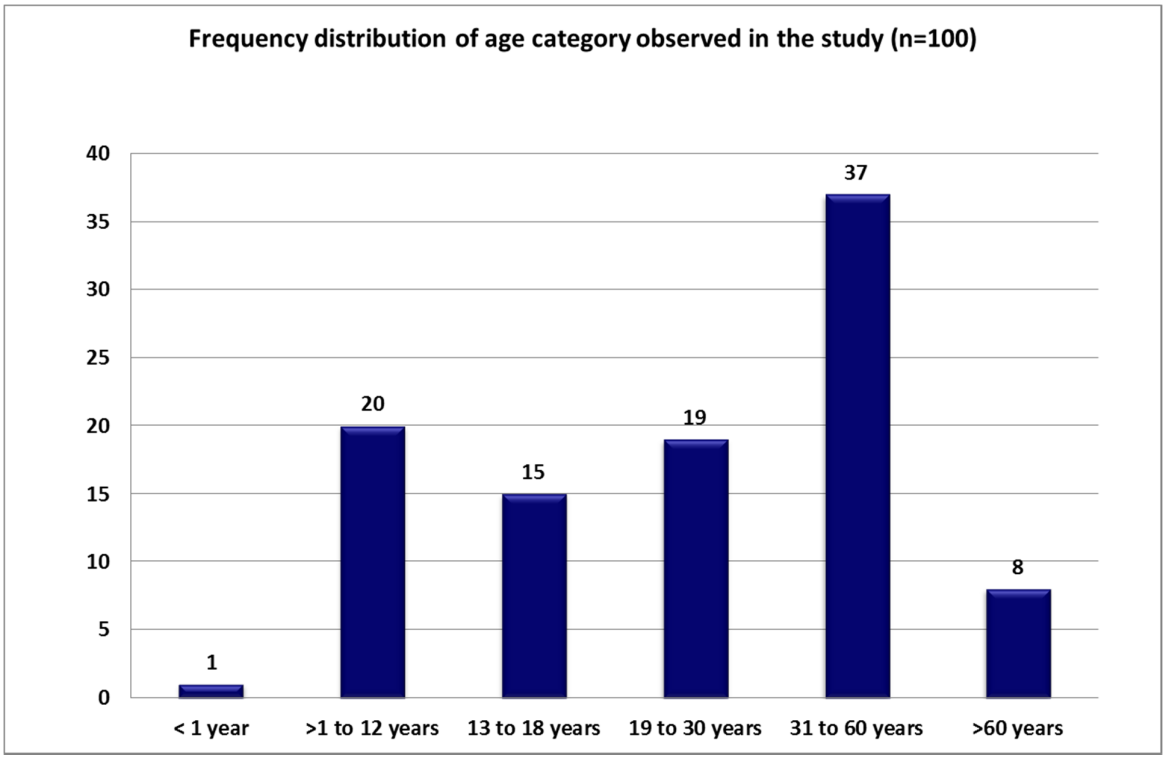

Fig 15: Frequency distribution of age category in our study.

This bar chart clearly explains that more common affected age group is 31-60 years (37%) followed by 1-12 years (21%) age group.

Table 2. Overall frequency distribution of gender in the study.

S.No	Gender	Frequency (n)	Proportion (%)
1	Male	54	54
2	Female	46	46

Data are expressed as n and %. The total N=100.

Percentage of males with CSOM was 54% whereas the percentage of female was 46%

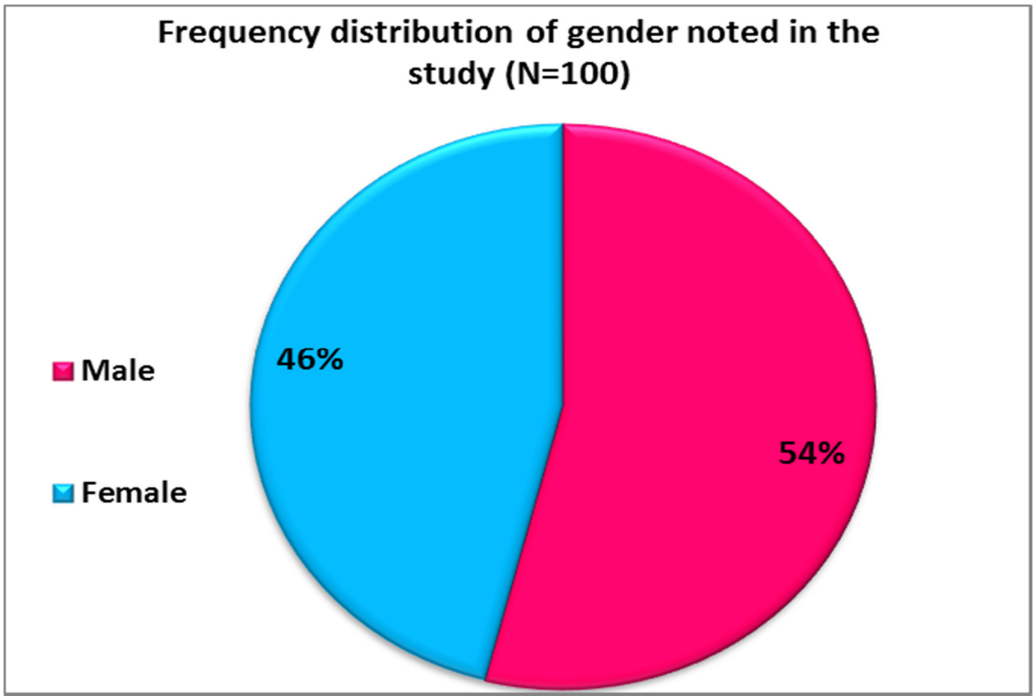

Fig 16: Frequency distribution of gender in the study.

This pie chart shows there was a preponderance of male patients accounts for 54% followed by female (46%)

Table 3. Overall frequency distribution of age population in the study.

S.No	Age Population	Frequency (n)	Proportion (%)
1	Adult	78	78
2	Pediatrics	22	22

Data are expressed as n and %. The total N=100.

Out of 100 cases, majority belongs to adult age group (78%) followed by pediatric age group (22%)

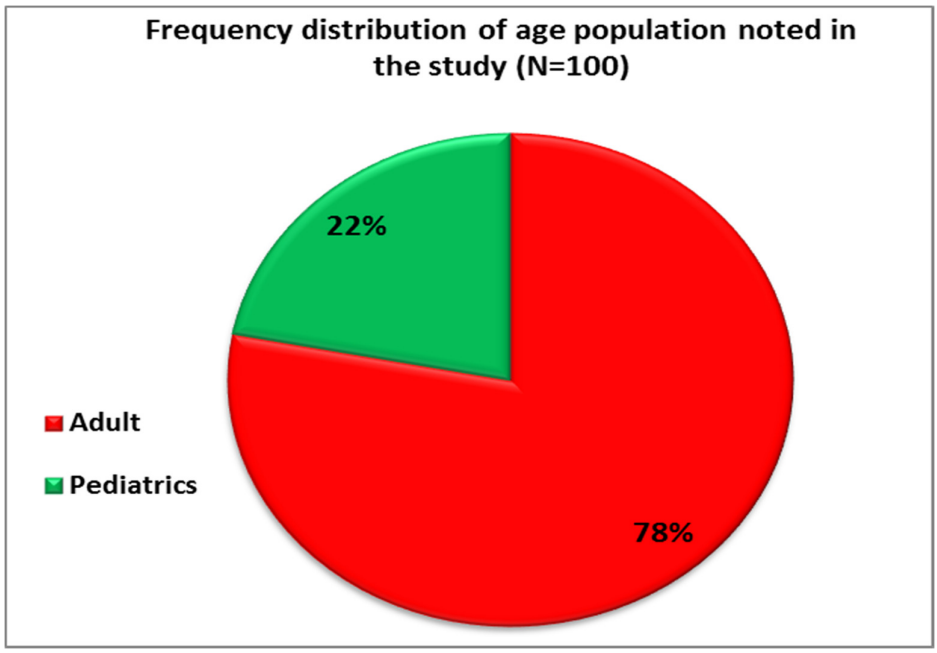

Fig 17: Frequency distribution of age population.

This diagram illustrates that 78% belongs to adult age group and 22% are pediatric age groups.

Table 4. Overall frequency distribution of laterality of CSOM in the study.

S.No	Laterality	Frequency (n)	Proportion (%)
1	Bilateral	22	22
2	Unilateral-right	39	39
3	Unilateral -Left	39	39

Data are expressed as n and %. The total N=100.

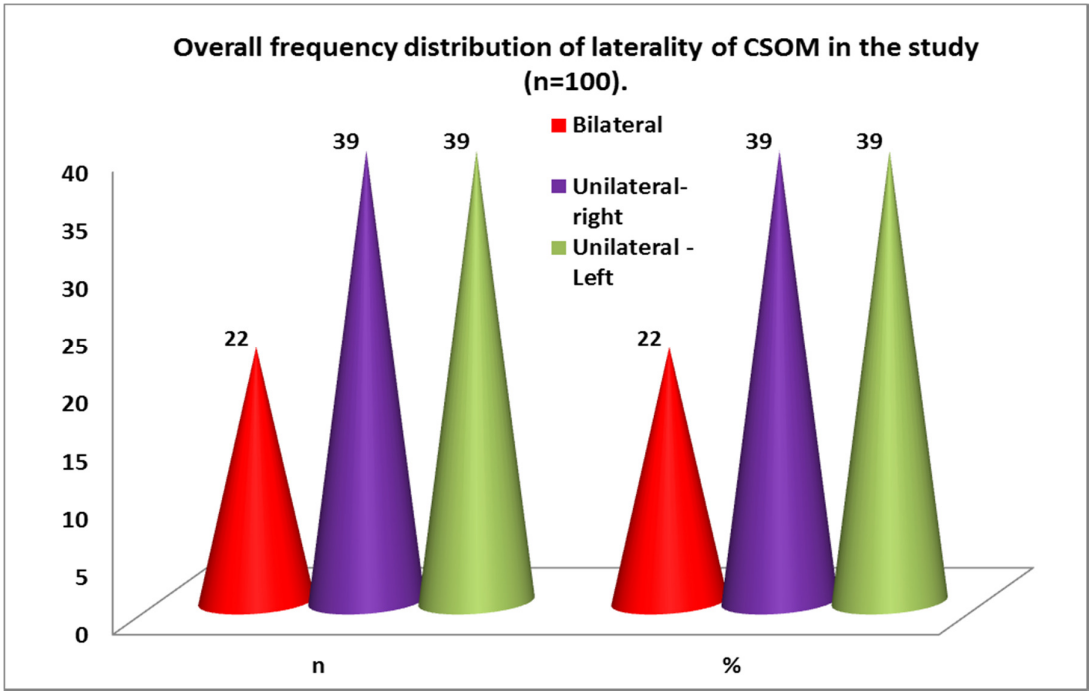

Fig 18: Frequency distribution of laterality of CSOM in the study.

According to laterality, majority (78%) suffered from unilateral CSOM whereas 22% suffered from bilateral CSOM.

Table 5. Overall frequency distribution of growth observed in CSOM samples in the study.

S.No	Nature of growth	Frequency (n)	Proportion (%)
1	Growth present	91	91
2	Growth absent	9	9

Data are expressed as n and %. The total N=100.

Among 100 patients, culture positivity were 91%. Remaining 9% were culture negative.

Table 6. Overall frequency distribution of nature of organism isolated in CSOM samples in the study.

S.No	Nature of growth	Frequency (n)	Proportion (%)
1	Gram negative bacilli	56	56
2	Gram positive cocci	27	27
3	Fungal	7	7
4	Gram negative cocco-bacilli	1	1
5	No growth	9	9

Data are expressed as n and %. The total N=100.

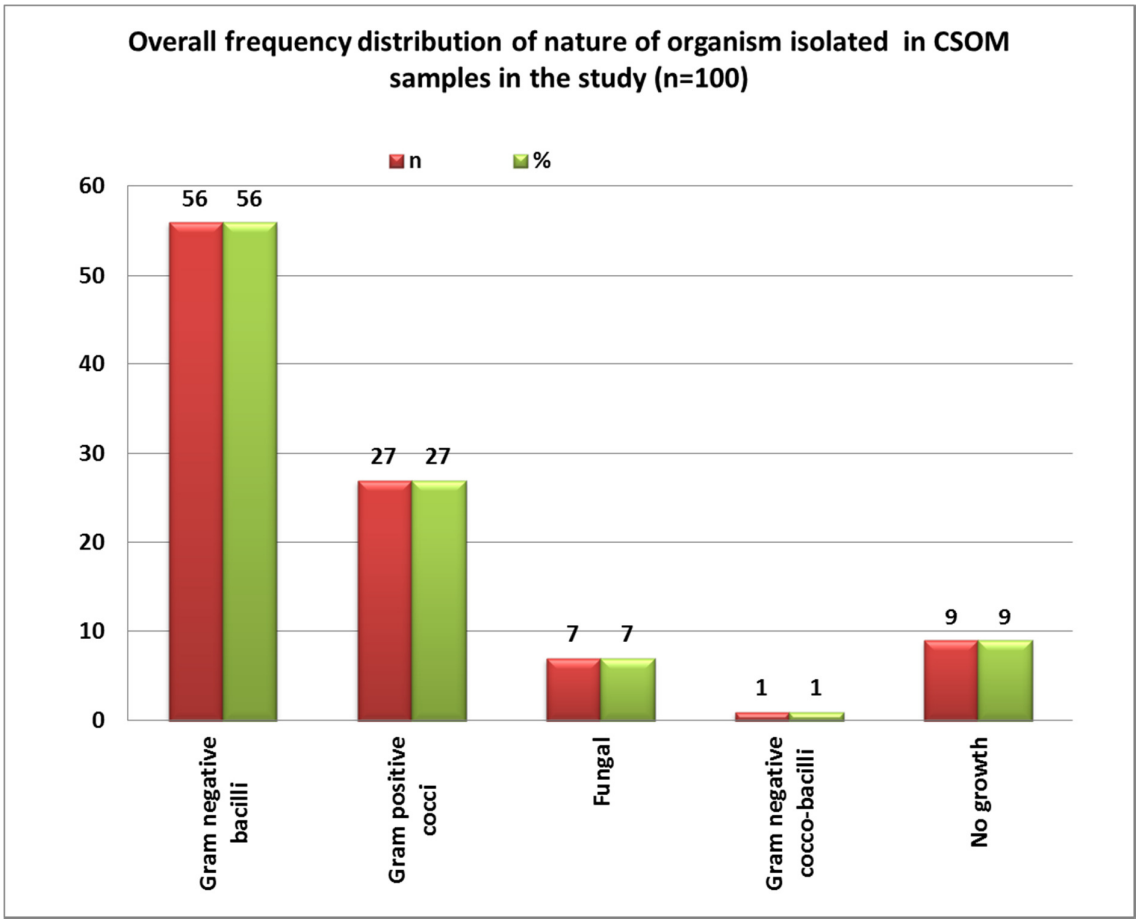

Fig 19: Frequency distribution of nature of organism isolated in CSOM

This bar diagram clearly explains that in our study, gram negative bacilli (56%) was more common compared to gram positive cocci (27%). Fungal isolate was 7%. Only one isolate of gram negative coccobacilli was isolated in this study.

Table 7. Comparison of frequency distribution of gender with respect to age population in the study.

S.No	Gender category	Adult group (n=78)		Pediatric group (n=22)		Chi-square	p value
		n	%	n	%		
1	Male	36	46.2	18	81.8*	8.787	0.003*
2	Female	42	53.8*	4	18.2		

Data are expressed as n with %. Fisher's exact test was used to compare the frequencies between the groups. *indicates p<0.05 and considered significant.

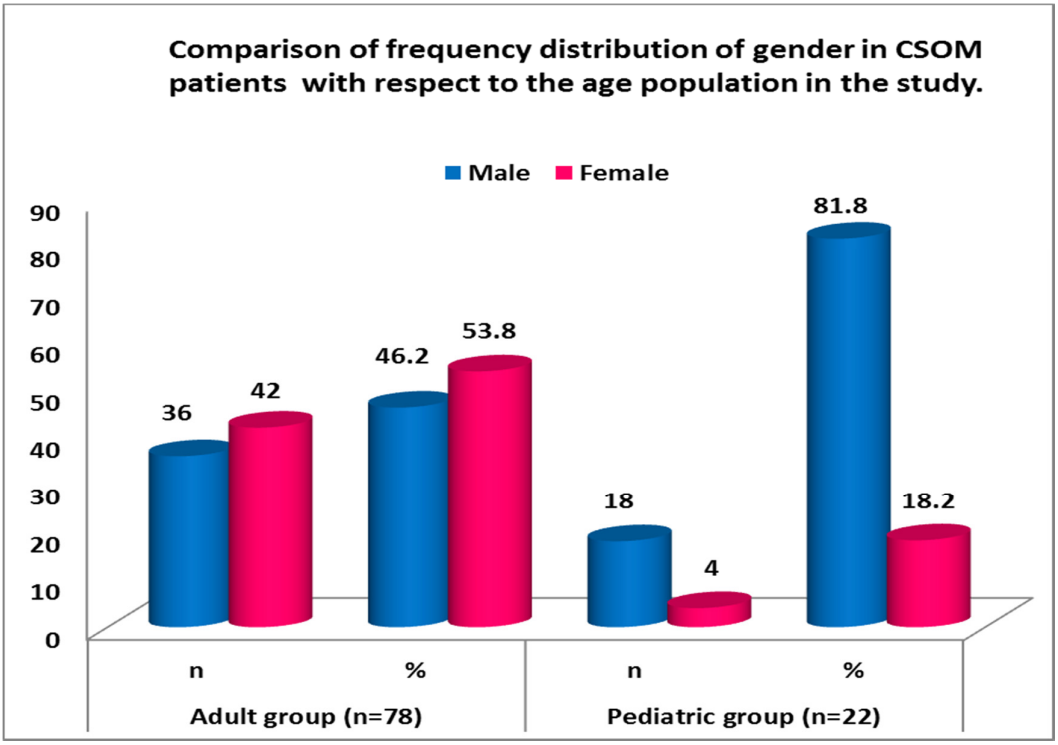

Fig 20: Comparison of frequency distribution of gender in CSOM with respect to age population in the study.

Table 8. Comparison of frequency distribution of laterality of CSOM with respect to the age population in the study.

S.No	Laterality of CSOM	Adult group (n=78)		Pediatric group (n=22)		Chi-square	p value
		n	%	n	%		
1	Bilateral	12	15.4	10	45.5*	9.04	0.007*
2	Unilateral	66	84.6*	12	54.5		

Data are expressed as n with %. Fisher's exact test was used to compare the frequencies between the groups. *indicates $p<0.05$ and considered significant.

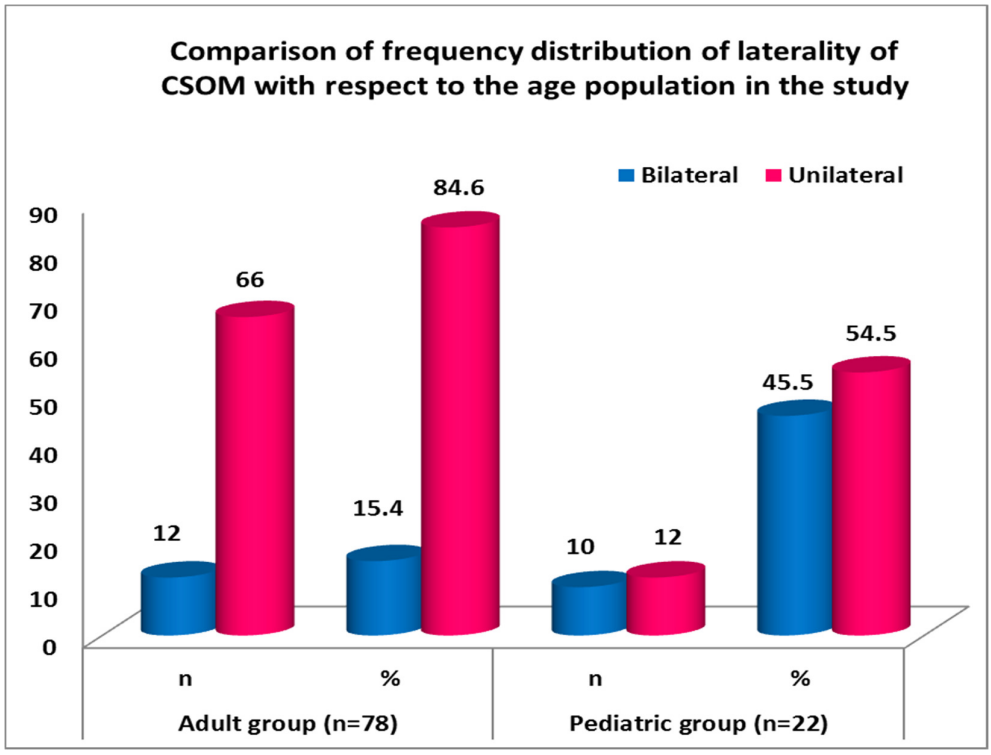

Fig 21: Comparison of frequency distribution of laterality of CSOM with respect to the age population in the study.

Table 9. Comparison of frequency distribution of occurrence of growth in culture from CSOM sample with respect to the age population in the study.

S.No	Type of growth	Adult group (n=78)		Pediatric group (n=22)		Chi-square	p value
		n	%	n	%		
1	Growth present	70	89.7	21	95.5	0.683	0.679 (NS)
2	No growth	8	10.3	1	4.5		

Data are expressed as n with %. Fisher's exact test was used to compare the frequencies between the groups. NS = Not significant.

Table 10. Comparison of nature of organism isolated from CSOM sample with respect to age population in the study.

S.No	Nature of organism	Adult group (n=78)		Pediatric group (n=22)		Chi-square	p value
		n	%	N	%		
1	Gram negative bacilli	43	55.1	13	59.1	1.**129**	0.890 (NS)
2	Gram positive cocci	21	26.9	6	27.3		
3	Fungal	5	6.4	2	9.1		
4	Gram negative cocco-bacilli	1	1.3	0	0		
5	No growth	8	10.3	1	4.5		

Data are expressed as n with %. Fisher's exact test was used to compare the frequencies between the groups. NS = Not significant.

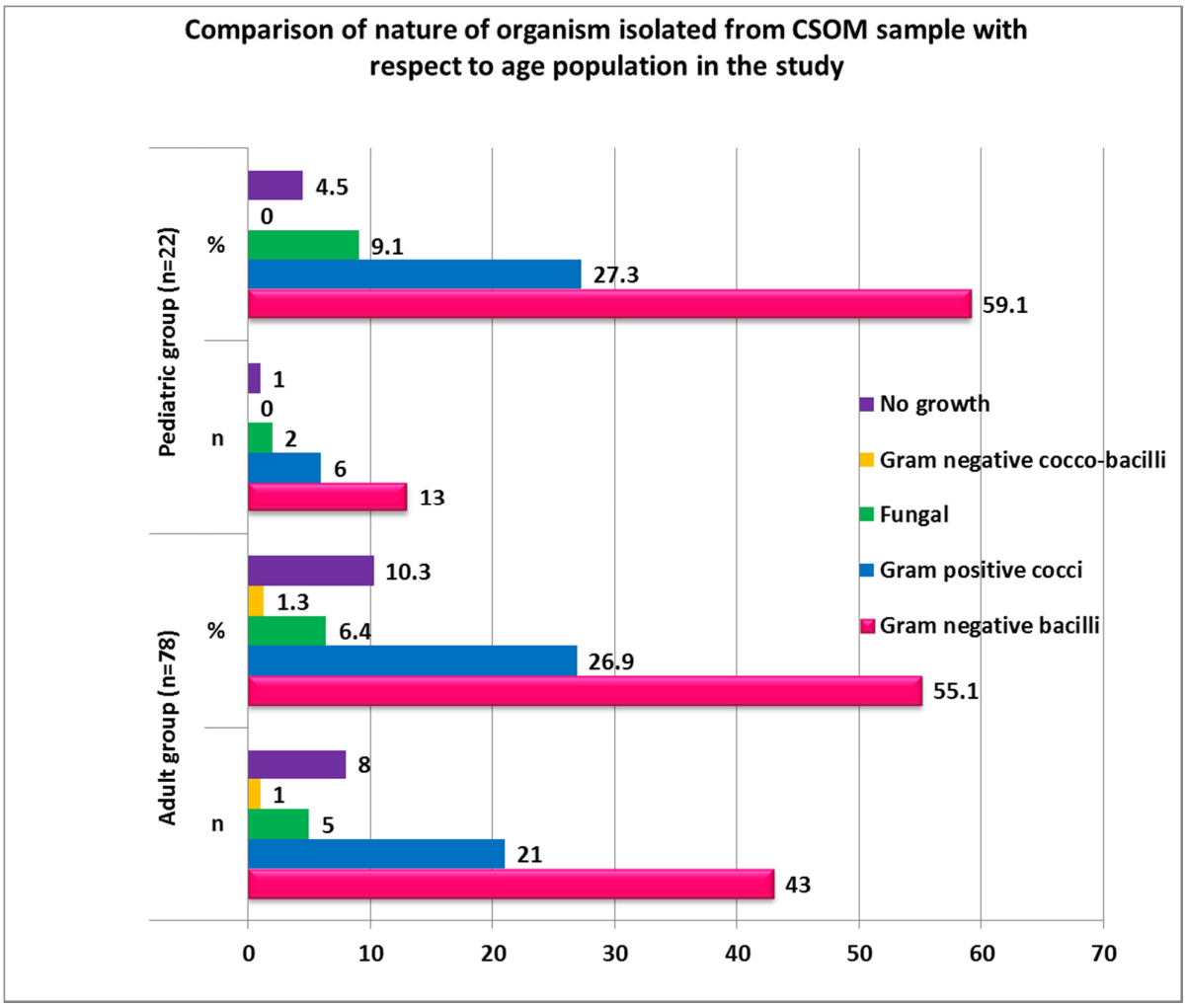

Fig 22: Comparison of nature of organism isolated from CSOM sample with respect to age population in the study.

Table 11. Comparison of nature of organism isolated from CSOM sample with respect to laterality in the study.

S.No	Nature of organism	Bilateral (n=22)		Unilateral (n=78)		Chi-square	p value
		n	%	n	%		
1	Gram negative bacilli	12	54.5	44	56.4	3.29	0.416 (NS)
2	Gram positive cocci	8	36.4	19	24.4		
3	Fungal	2	9.1	5	6.4		
4	Gram negative cocco-bacilli	0	0	1	1.3		
5	No growth	0	0	9	11.5		

Data are expressed as n with %. Fisher's exact test was used to compare the frequencies between the groups. NS = Not significant.

Table 12. Frequency distribution of organisms isolated from the growth in the study population.

S.No	Name of the organism Isolated from the culture		Total growth (n=91)	
			n	%
1	Gram Negative Bacilli	Pseudomonas aeruginosa	32	35.2
2		Klebsiella pneumonia	13	14.3
3		Escherichia coli	3	3.3
4		Proteus	6	6.6
5		Enterobacter	1	1.1
6		Citrobacter	1	1.1
7	Gram Positive Cocci	Staphylococcus aureus	23	25.3
8		CONS	2	2.2
9		Enterococci	2	2.2
10	Gram negative coccobacilli	Acinetobacter	1	1.1
11	Fungal	Candida species	3	3.3
12		Aspergillus flavus	2	2.2
13		Aspergillus niger	2	2.2

Data are expressed as n with %. The total N=91.

Table 13. Frequency distribution of drug sensitivity or resistance of Acinetobacter to various antibiotics in the study population.

S.No	Drug Name	Acinetobacter isolated (n=1)			
		Sensitive		Resistant	
		n	%	n	%
1	Co-trimoxazole	1	100	0	0
2	Amikacin	0	0	1	100
3	Gentamicin	0	0	1	100
4	Ciprofloxacin	0	0	1	100
5	Ceftazidime	1	100	0	0
6	Meropenem	1	100	0	0
7	Piperacillin+Tazobactam	1	100	0	0

Data are expressed as n with %.

In our study, Acinetobacter, Gram negative cocco bacilli, shows 100% sensitive to ceftazidime, cotrimoxazole, meropenem and piperacillin-tazobactam. It also shows 100% resistant to amikacin, gentamicin and ciprofloxacin.

Table 14. Frequency distribution of drug sensitivity or resistance of Citrobacter to various antibiotics in the study population.

S. No	Drug Name	Citrobacter isolated (n=1)			
		Sensitive		Resistant	
		n	%	n	%
1	Ampicillin	1	100	0	0
2	Co-trimoxazole	1	100	0	0
3	Amikacin	1	100	0	0
4	Gentamicin	1	100	0	0
5	Cefotaxime	1	100	0	0
6	Meropenem	1	100	0	0
7	Piperacillin-Tazobactam	1	100	0	0

Data are expressed as n with %.

Citrobacter shows sensitive to all drugs like ampicillin, cotrimoxazole, amikacin, gentamicin, cefotaxime, meropenem and piperacillin-tazobactam.

Table 15. Frequency distribution of drug sensitivity or resistance of CONS to various antibiotics in the study population.

S. No	Drug Name	CONS isolated (n=2)			
		Sensitive		Resistant	
		n	%	n	%
1	Ampicillin	1	50	1	50
2	Erythromycin	1	50	1	50
3	Clindamycin	1	50	1	50
4	Doxycycline	2	100	0	0
5	Co-trimoxazole	0	0	2	100
6	Cefoxitin	2	100	0	0
7	Linezolid	2	100	0	0
8	Ciprofloxacin	1	50	1	50

Data are expressed as n with %.

Coagulase negative Staphylococcus shows 100% sensitive to doxycycline, cefoxitin and linezolid and 100% resistant to Cotrimoxazole.

Table 16. Frequency distribution of drug sensitivity or resistance of Enterobacter to various antibiotics in the study population.

S.No	Drug Name	Enterobacter isolated (n=1)			
		Sensitive		Resistant	
		n	%	n	%
1	Co-trimoxazole	1	100	0	0
2	Amikacin	1	100	0	0
3	Gentamicin	1	100	0	0
4	Ciprofloxacin	1	100	0	0
5	Cefotaxime	1	100	0	0
6	Meropenem	1	100	0	0
7	Piperacillin+Tazobactam	1	100	0	0

Data are expressed as n with %.

Enterobacter exhibits 100% sensitive to all drugs like amikacin, gentamicin, ciprofloxacin, cefotaxime, cotrimoxazole, meropenem and pip-taz.

Table 17. Frequency distribution of drug sensitivity or resistance of Enterococci to various antibiotics in the study population.

S.No	Drug Name	Enterococci isolated (n=2)			
		Sensitive		Resistant	
		n	%	N	%
1	Ampicillin	1	50	1	50
2	High level gentamicin	2	100	0	0
3	Vancomycin	2	100	0	0
4	Linezolid	2	100	0	0
5	Ciprofloxacin	0	0	2	100

Data are expressed as n with %.

Enterococci reveals that 100% sensitive to High level gentamicin, vancomycin and linezolid. 50% sensitive to ampicillin and ciprofloxacin.

Table 18. Frequency distribution of drug sensitivity or resistance of E. coli to various antibiotics in the study population.

S. No	Drug Name	E. coli isolated (n=3)			
		Sensitive		Resistant	
		n	%	n	%
1	Ampicillin	3	100	0	0
2	Co-trimoxazole	2	66.6	1	33.4
3	Amikacin	3	100	0	0
4	Gentamicin	2	66.6	1	33.4
5	Ciprofloxacin	1	33.4	2	66.6
6	Cefotaxime	3	100	0	0
7	Meropenem	3	100	0	0
8	Piperacillin+Tazobactam	3	100	0	0

Data are expressed as n with %.

Escherichia coli shows 100% sensitive to cefotaxime, ampicillin, meropenem and pip-taz. 66.6% sensitive to gentamicin, cotrimoxazole. And 33.4% sensitive to ciprofloxacin.

Table 19. Frequency distribution of drug sensitivity or resistance of Klebsiella pneumoniae to various antibiotics in the study population.

S. No	Drug Name	Klebsiella isolated (n=13)			
		Sensitive		Resistant	
		n	%	N	%
1	Co-trimoxazole	9	69.2	4	30.8
2	Amikacin	11	84.6	2	15.4
3	Gentamicin	8	61.5	5	38.5
4	Ciprofloxacin	7	53.8	6	46.2
5	Cefotaxime	9	69.2	4	30.8
6	Meropenem	13	100	0	0
7	Piperacillin+Tazobactam	13	100	0	0

Data are expressed as n with %.

Klebsiella shows that 100% sensitive to meropenem and pip-taz. Also, it exhibits 69.2% sensitive to both cotrimoxazole and cefotaxime. 84.6% and 61.5% sensitivity to amikacin and gentamicin respectively.

Table 20. Frequency distribution of drug sensitivity or resistance of proteus to various antibiotics in the study population.

S. No	Drug Name	Proteus isolated (n=6)			
		Sensitive		Resistant	
		n	%	n	%
1	Co-trimoxazole	4	66.7	2	33.3
2	Amikacin	6	100	0	0
3	Gentamicin	5	83.3	1	16.7
4	Ciprofloxacin	2	33.3	4	66.7
5	Cefotaxime	6	100	0	0
6	Meropenem	6	100	0	0
7	Piperacillin+Tazobactam	6	100	0	0

Data are expressed as n with %.

Proteus exhibits that 100% sensitive to amikacin, cefotaxime, meropenem and pip-taz. 83.3% and 66.7% sensitivity to gentamicin and cotrimoxazole respectively.

Table 21. Frequency distribution of drug sensitivity or resistance of Pseudomonas to various antibiotics in the study population.

S.No	Drug Name	Pseudomonas isolated (n=32)			
		Sensitive		Resistant	
		n	%	n	%
1	Amikacin	31	96.9	1	3.1
2	Gentamicin	24	75	8	25
3	Ciprofloxacin	28	87.5	4	12.5
4	Ceftazidime	32	100	0	0
5	Meropenem	32	100	0	0
6	Piperacillin+Tazobactam	32	100	0	0

Data are expressed as n with %.

Pseudomonas aeruginosa shows that 100% sensitive to ceftazidime, meropenem and pip-taz. It also shows that 96.9%, 87.5%, 75% sensitivity to amikacin, ciprofloxacin and gentamicin respectively.

Table 22. Frequency distribution of drug sensitivity or resistance of Staph aureus to various antibiotics in the study population.

S.No	Drug Name	S.aureus isolated (n=23)			
		Sensitive		Resistant	
		n	%	N	%
1	Ampicillin	8	34.8	15	65.2
2	Erythromycin	15	65.2	8	34.8
3	Clindamycin	15	65.2	8	34.8
4	Doxycycline	13	56.5	10	43.5
5	Co-trimoxazole	16	69.6	7	30.4
6	Cefoxitin	14	60.9	9	39.1
7	Linezolid	23	100	0	0
8	Ciprofloxacin	16	69.6	7	30.4

Data are expressed as n with %.

Coming to Staphylococcus aureus, it reveals 100%, 60.9%, 56.5%, 34.8% sensitivity to linezolid, cefoxitin, doxycycline and ampicillin respectively. Out of 23 isolates, 9 isolates are (i.e.39.1%) resistant to cefoxitin, hence it is considered as methicillin resistant Staphylococcus aureus.

Table 23: Vancomycin susceptibility by vancomycin agar screen method
All MRSA strains were susceptible to vancomycin.

S.No	Name of the isolate	N	Name of the resistant pattern	n	Vancomycin Susceptibility
1.	Staphylococcus aureus	23	MRSA	9	Susceptible

All MRSA strains (9) were susceptible to vancomycin.

MOLECULAR CHARACTERISATION RESULTS BY GENOTYPING

Among 23 isolates of Staphylococcus aureus, 9 isolates were positive for MRSA, detected by cefoxitin disc diffusion method. Conventional PCR was done to evaluate the molecular characterization.

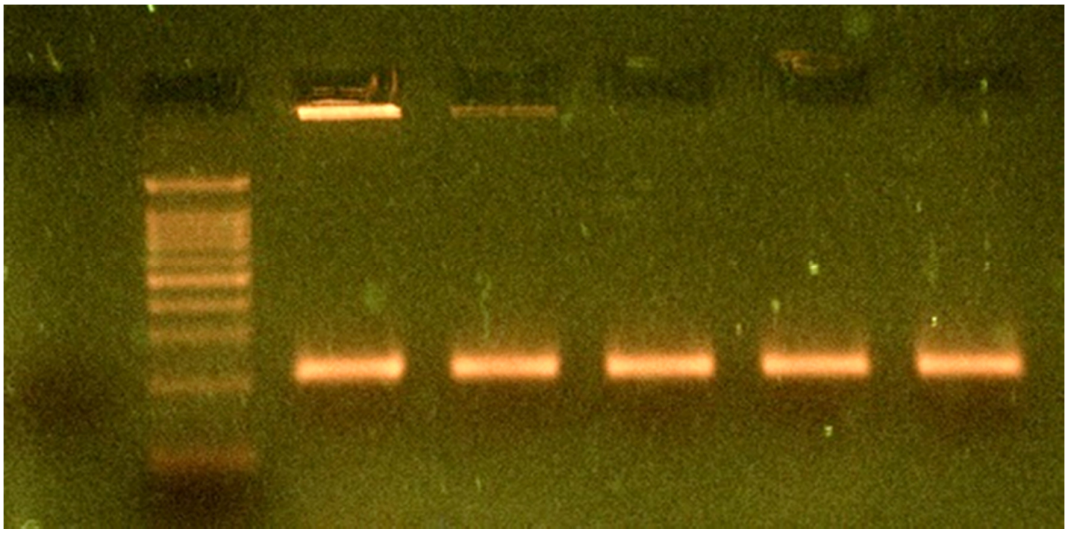

Fig 23: mec A gene detection by Conventional PCR

Method	Total sample of Methicillin Resistant Staph aureus	Positive for mec A gene	Negative for mec-A gene
Conventional PCR	9	9	0

DISCUSSION

This prospective study was conducted at Thanjavur Medical College Hospital in association with the Department of Ear, Nose and Throat. The study population consisted of 100 patients with signs and symptoms of ear discharge, ear pain and fever. Out of 100 cases, majority (37%) were in 31-60yrs of age group which is in concordance with the findings of various other studies Faiz Farhan et al[8] while Pallawi Goyal et al[25], Susmita Kumari Sahu et al[14], Mahajan et al[55] and Kaur P et al[26] reported higher incidence of CSOM in the 11-20yrs of age group.

In this study there was a preponderance of male patients accounting for 54%. It is similar to study by Bansal Sulabh et al[35] in which male were 62.1% & female were 37.9%. Our study is closely related to Susmita kumari sahu et al[14] in which male were 58% & female were 42%

Occurrence of unilateral and bilateral disease was in the ratio of 3:1(78% and 22% respectively). Deshmukh KA et al[27] observed 35.6% left ear, 48.2% right ear & bilateral was 16%.

Pseudomonas aeruginosa was the most prevalent organism from CSOM cases reported in several studies [26,30,31,32,35,55,56]

Aparna Chavan et al[32] study showed that Pseudomonas aeruginosa was the commonest isolate followed by Proteus mirabilis. Rajat Prakash et al[20] denoted Staphylococcus aureus was the most common isolate followed by

Pseudomonas aeruginosa. It is contrast to our study, in which Pseudomonas aeruginosa was the commonest isolate followed by Staphylococcus aureus.

Isolated organism

Among the 100 patients, culture positivity was 91%. It is similar to study done by Alphonse Bizimana et al[42] (94.6%)

In this study gram negative bacilli was more common (56%) compared to gram positive cocci (27%). Fungal isolate was 7%. It is similar to study done by Kaur P et al[26] in which gram negative bacilli were more common. In Gram negative bacilli, culture positive cases include Pseudomonas aeruginosa (35.2%), Klebsiella pneumoniae (14.3%), Proteus species (6.6%), Escherichia coli (3.3%), Enterobacter (1.1%), Citrobacter (1.1%) & Acinetobacter (1.1%). In gram positive cocci, culture positive cases include Staphylococcus aureus (25.3%), CONS (2.2%) & Enterococci (2.2%). Among the fungal isolates, Candida species (3.3%), Aspergillus flavus (2.2%) & Aspergillus niger (2.2%). This is similar to a study done by Susmita kumari sahu et al[14] in which candida species were more common followed by aspergillus species.

Among the 100 CSOM patients, Unilateral ear discharge was the most common presentation in both adults (84.6%) and paediatric age groups (54.5%).

Among the 100 CSOM patients, female were more commonly affected in adult age groups (53.8%), whereas in paediatric age groups (81.8%) male were commonly affected.

In adult age groups (78%), the most common isolated pathogen are gram negative bacilli (55.1%) followed by gram positive cocci (26.9%), fungus (6.4%), gram negative coccobacilli (1.3%).

In pediatric age group (22%), the most common isolated pathogen are gram negative bacilli (59.1%) followed by gram positive cocci (27.3%), fungus (9.1%).

ANTIBIOTIC SUSCEPTIBILITY

The commonest bacteria isolated in this study was Pseudomonas aeruginosa (35.2%) which showed 100% sensitive to ceftazidime, meropenem and pip-taz. It also showed 96.9% sensitive to amikacin, 87.5% sensitive to ciprofloxacin and 75% sensitive to gentamicin (table 21)

Next most common isolate was Staphylococcus aureus (25.3%) which showed 100% sensitive to linezolid, 69.6% sensitive to cotrimoxazole and ciprofloxacin, 65.2% sensitive to erythromycin and clindamycin, 60.9% sensitive to cefoxitin and 56.5% sensitive to doxycycline (table 22)

Klebsiella pneumoniae showed 100% sensitive to meropenem and pip-taz. 84.6% sensitive to amikacin. 69.2% sensitive to cefotaxime and

cotrimoxazole. 61.5% sensitive to gentamicin and 53.8% sensitive to ciprofloxacin (table 19)

Proteus showed 100% sensitive to meropenem, pip-taz, cefotaxime and amikacin. It showed 83.3% sensitive to gentamicin, 66.7% sensitive to co trimoxazole and 33.3% sensitive to ciprofloxacin (table 20)

E coli showed 100% sensitive to ampicillin, cefotaxime, amikacin, meropenem and pip-taz. 66.6% sensitive to gentamicin and cotrimoxazole. 33.4% sensitive to ciprofloxacin. (table 18)

Enterobacter showed 100 % sensitive to all drug such as amikacin, gentamicin, ciprofloxacin, cotrimoxazole, cefotaxime, meropenem and pip-taz (table 16)

Citrobacter showed 100% sensitive to amikacin, gentamicin, ampicillin, cotrimoxazole, cefotaxime, meropenem and pip-taz (table 14)

Among the gram-positive cocci, CONS showed 100% sensitive to linezolid, cefoxitin and doxycycline. 50% sensitive to ampicillin, erythromycin, clindamycin and ciprofloxacin (table 15)

Enterococcus species showed 100% sensitive to high level gentamicin, vancomycin and linezolid. 50% sensitive to ampicillin. (table 17)

Antimicrobial resistance pattern

Among the Staphylococcus aureus isolates, 60.9% showed sensitive to cefoxitin and 39.1% showed resistant to cefoxitin. Out of 23 isolates, 14 isolates (60.9%) were MSSA and 9 isolates (39.1%) were MRSA.

Among the Klebsiella species (14.3%), 4 isolates (30.8%) were showed resistant to third generation cephalosporin (i.e. cefotaxime), and they were considered as ESBL positive klebsiella.

SUMMARY

This study was conducted at the thanjavur medical college and hospital, thanjavur. 100 pus samples were collected in CSOM patients to evaluate the microbiological profile and antimicrobial susceptibility pattern.

1. Ear discharge was the most common presenting symptom.
2. Majority of the patients belong to 31-60yrs (adults)
3. Males were more commonly affected than females.
4. CSOM with unilateral presentation were more common than bilateralism.
5. The pus culture positivity rate was 91%. The spectrum of bacterial isolates were 56% for GNB 27% for GPC and 7% for fungal isolates.
6. Pseudomonas aeruginosa (35.2%) was the most common isolate followed by Staphylococcus aureus (25.3%). Among the staphylococcus aureus, 39.1% were MRSA producers. mec A gene are responsible for resistant Staphylococci and was detected by molecular genotyping method (Polymerase Chain Reaction)

CONCLUSION

A total of 100 pus samples were collected in our study and processed at the department of microbiology, Thanjavur Medical College, Thanjavur. This study estimates the prevalence of bacterial isolate and fungal isolate in pus sample of CSOM patients in a tertiary care hospital. Bacterial infections are the most common pathogen in our study among which Pseudomonas aeruginosa and Staphylococcus aureus were the commonest. MRSA (39.1%) was isolated and screened by phenotypic method (disc diffusion test). It was confirmed by genotypic method using PCR and the responsible gene (mec A) causing the resistance was identified. Apart from MRSA, other resistant strains such as ESBL were also identified among gram negative bacilli.

Thus, we conclude that, there is an alarming rise of antibiotic resistance among the isolated strains. To control MRSA, simple hand washing with soap containing disinfectants and use of personal protective equipment like gloves, masks should be followed. Mupirocin, a topical antibiotic are to be used to prevent staphylococcal colonisation in nasal carriers.

As per CLSI 2019 guidelines, recommend ESBL testing for epidemiological infection control purposes only. Routine ESBL testing of clinical isolates are no longer necessary. Presence of ESBL and MRSA strains are leading cause of morbidity and mortality. Irrational use of antibiotics leads

to multi drug resistant in the community. All higher antibiotic should be prescribed only after getting approval from the infection control committee. To reduce the morbidity and mortality, the following measures are followed.

1. Early detection of CSOM and proper guidelines for antibiotic therapy.
2. Choice of an antibiotics should be based on local pathogens prevalence and antimicrobial susceptibility pattern.
3. Implementation of infectious control measures to reduce the disease burden in the community.
4. Health education.

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ABBREVATIONS

CSOM – Chronic Suppurative Otitis Media

ATCC – American Type Culture Collection

CLSI – Clinical & Laboratory Standards Institute.

GNB – Gram Negative Bacilli.

GPC – Gram Positive Cocci.

URTI – Upper Respiratory Tract Infection.

ESBL – Extended Spectrum Beta Lactamase

MBL – Metallo Beta Lactamase

MRSA – Methicillin Resistant Staph aureus.

MIC – Minimum Inhibitory Concentration.

AST – Antimicrobial Susceptibility Test

MHA – Muller Hinton Agar.

SDA – Sabouraud's Dextrose Agar.

LPCB – Lactophenol Cotton Blue

Ak – Amikacin

Gm – Gentamicin.

Cip – Ciprofloxacin.

Ery – Erythromycin.

Pen – Pencillin.

Cx – Cefoxitin.

CoT – Cotrimoxazole.

CAZ – Ceftazidime

Mero – Meropenem

PT – Piperacillin Tazobactam

IMP – Imipenem

PROFORMA

Name: OP/IP No:

Father's Name: Micro Lab No:

Age: Date of sample collection:

Sex: Specimen:

Address: Test:

Father's Occupation:

Family income:

Chief Complaints:

1. Fever
2. Ear discharge
3. Ear pain

Past History:

Previous episodes

Personal history of smoking

Occupational history

History of Co-Morbid illness

Family history

Clinical diagnosis

Differential diagnosis

Physical examination

CONSENT FORM

STUDY TITLE

" A PREVALENCE STUDY OF MICROBIOLOGICAL PROFILE IN PUS SAMPLES OF CHRONIC SUPPURATIVE OTITIS MEDIA PATIENTS, DRUG RESISTANCE PATTERN AND ITS MOLECULAR CHARACTERISATION AT THANJAVUR MEDICAL COLLEGE AND HOSPITAL, THANJAVUR.

I ……. Hereby give consent to participate in the study conducted by Dr.B.SHANTHI BHUSHANA, post graduate in Thanjavur Medical College, Thanjavur to use my personal clinical data and the results of investigations for the purpose of analysis and to study the nature of the disease. I also give consent to give my pus sample for further investigations. I also learn that there is no additional risk in this study. I also give my consent to the investigator to publish the data in any form or journal.

Signature / Thumb impression of the patient/ relative Place:

Date:

Name of patient:

Address of patient:

Signature of investigator:

Signature of guide