CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1. Background

Otitis media or middle ear infection is the inflammation of the mucosal lining of the middle ear including the tympanic membrane. The Seventh Research committee formed after the Seventh International Symposium on otitis media broadly classified the infection into Acute Otitis Media (AOM); Otitis Media with Effusion (OME) and its related disorder; and Eustachian tube dysfunction (Bluestone *et al.,* 2007). Similarly, intratemporal complications and intracranial complications are the types of complications and sequele of otitis media (Bluestone *et al.,* 2007),

Suppurative infection of middle ear with sudden onset is said to be AOM. Inflamed mucoperiosteal lining of the middle ear makes the condition clinically identifiable (Maxson *et al.*, 1996). The major symptoms are otalgia, fever, irritability, restless sleep and diminished appetite (McWilliams *et al.*, 2011)

Clinical practice guideline of American Academy of Pediatrics defines OME as the presence of fluid in the middle ear without signs or symptoms of acute ear infection. OME is considered different from AOM as there is no acute onset of signs or symptoms or middle ear inflammation.

The World Health Organization (WHO) defines Chronic Suppurative Otitis Media (CSOM) as chronic infection of middle ear cleft with constant perforation of tympanic membrane and discharge for at least two weeks (WHO/CIBA, 1996). Synonyms for CSOM include chronic otitis media (without effusion), chronic mastoiditis, and chronic tympanomastoiditis (Acuin, 2007).

The infection may be unilateral (affecting only one ear) or bilateral (affecting both ears). Commonly, bilateral infection is seen among adults than in children and

may be caused by a single organism or multiple organisms. Even the unilateral infection may be due to only one organism or it may be polymicrobial. *Staphylococcus, Pseudomonas, Moraxella, Streptococcus* etc. are some of the few bacteria isolated from the patients suffering from otitis media.

The onset of ear infection is common in childhood. It is estimated that by 2 years of age, at least 90% of children would have experienced at least one episode of otitis media (Bluestone *et al.*, 2007). Children from developing countries like Nepal are more prone to the infection than those in developed countries. The major cause of this distinction is lower socio-economic status, poor hygiene and ignorance about health problems in the developing countries (Kumar *et al.*, 2011). The complications due to otitis media are also more frequent in these countries because of the same reasons.

The recommended management of AOM and OME is watchful waiting of patients for certain time, if the patients are not at risk (McWilliams *et al.*, 2011; Neff, 2004). This is because most of the cases of AOM have spontaneous resolution (McWilliams *et al.*, 2011) and in case of OME antimicrobials and corticosteroids do not have long-term efficacy (Neff, 2004). However, CSOM is frequently found to lead to serious complications which could be prevented by timely administration of appropriate antibiotics.

Thickening of the middle ear mucosa, mucosal polyps, and cholesteatoma can be the results of CSOM (Acuin, 2007). It can also cause erosion of walls of middle ear exposing facial nerve, which may further lead to facial nerve paralysis, lateral sinus thrombosis, labyrinthitis, meningitis and brain abscess (WHO, 2004). It has also been seen to be associated with sensorineural hearing loss (Raquib *et al.*, 2009). It has been found to elevate bone conduction threshold thus, reducing the ability to hear faint noises (Raquib *et al.*, 2009). Since, CSOM is of long duration and greater severity compared to AOM, and since most children can perform optimally only at louder auditory stimuli than adults, CSOM in children is likely to inhibit language and cognitive development (WHO 2004). It further leads to

poor scholastic performance in children and can occasionally lead to fatal intracranial infections and acute mastoiditis, especially in resource-poor country (Acuin, 2007).

The main reasons for the development of complications are late diagnosis and treatment of the disease, wrong use of antibiotics, negligence of the people, etc. A study by BRINOS in 1991 revealed that 1.5 million out of 19 million people (7.9%) in Nepal had eardrum pathology out of which 32% developed hearing impairments. This may be because 61% of people with ear problem didn't attend health care facilities though they were aware of the infection they have.

Several studies illustrated that common anti-infective agents are efficient against aerobic as well as anaerobic organisms (Brook, 2008). Amoxycillin and ciprofloxacin are the common antibiotics prescribed to the patients visiting ENT OPD with active ear discharge. However, haphazard use of antibiotics, without any prescriptions, and poor follow up of the patients have led to development of resistant strains of infective organisms.

Although, about 80% of all acute otitis media resolve spontaneously without treatment within 2 to 3 days (Thorne *et al*, 2009; Alberti, n.d.), AOM may progress to a number of suppurative complications including acute mastoiditis, sigmoid sinus thrombosis, and intracranial abscess, if not treated properly.

Transient, moderately severe hearing loss during the first months of life is frequently caused due to OME. Since, this period of life is critical for development of the auditory system, active treatment should be considered if spontaneous recovery does not occur. Otherwise, there would be delay in language acquisition or even worse, sensorineural hearing loss (Boudewyns *et al*, 2011). That means, healthy manpower will be lost to the disease that could be easily cured. If the infection is recognized early, disability-adjusted life-years (DALY) and even the number of people suffering disability could be minimized. Proper diagnosis and treatment of the infection can reduce the incidence of

disability. The main aim of the study is to isolate the common aerobic etiological agents of otitis media in the patients visiting an Out Patient Department (OPD) of a hospital, determine the antibiotic susceptibility pattern of those isolates.

The advent of new antibiotics has changed the pattern of bacterial isolates causing ear infection. Administration of 7-valent pneumococcal conjugate vaccine (PCV-7) in children has reversed the order of frequency of isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae.* Previously, *Strep. pneumoniae* were isolated in higher frequency but now the number of *H. influenzae* isolates is greater (McWilliams *et al*, 2011). Moreover, improper use of antimicrobials has resulted in the rapid rise of resistant organisms. So, the general anti-microbial agent, who is active against certain organism in other part of the world, may not be effective in Nepal. It is, hence, important to determine the pattern of modern day isolates from ear infection and deduce the organism's antibiotic susceptibility pattern so that it would be easier for the clinicians to plan a general outline of treatment for patients with discharging ear. Our study will target to draw a general picture of the common causative organisms of otitis media and their susceptibility patterns and help to further the knowledge in the microbiology of this disease. It will also determine the efficiency of commonly used antibiotics in inhibiting the isolates.

1.2 Study objectives

General objective:

To identify the bacterial agents causing otitis media and assess their antibiotic susceptibility pattern.

Specific objectives:

1. To the bacterial pathogen from the ear discharge of the patients with the otitis media.

2. To determine the frequency of Gram positive and Gram negative organisms in otitis media cases.
3. To assess antibiotic susceptibility pattern of the isolates.

CHAPTER II

LITERATURE REVIEW

2.1 Otitis media

Otitis media or middle ear infection is the inflammation of the mucosal lining of the middle ear including the tympanic membrane (Alberti, n.d.). AOM, OME, CSOM are the different types of otitis media. Ear discharge is common in AOM (if the tympanic membrane is perforated) and CSOM cases. *Streptococcus pneumoniae, Strep. pyogenes, Haemophilus influenzae, Staph. aureus, Ps. aeruginosa,* etc are the common bacterial pathogens causing otitis media.

Otitis media is one of the common reasons of pediatrics visit in the United States (Laufer *et. al,* 2011). The situation is same for the developing country like Nepal. According to WHO, Country Health System Profile, ear infections occupies seventh position among the ten leading diseases in Nepal (1999-2000) with 1.3 total new visits as a percentage of total population.

Occurrence of otitis media in young children during the first six years of life is much higher, with peak at around age 2 (WHO, 2004). It is the most common mucosal infection observed in the children, after common cold (Bluestone *et al.*, 2007). The incidence of otitis media decreases during and after adolescence due to development of eustachian tube and increased immunity (Oni *et al.,* 2002). Nonetheless, it is one of the major ENT problems in adults. CSOM is the most frequent diagnosis in adults followed by AOM and otitis externa (Oni *et al.,* 2002).

2.2 Causative organisms of otitis media

The major pathogens causing acute otitis media are *H. influenzae* (48.0%), *S. pneumonia* (42.9%), *M. catarralis* (4.8%) and Group A streptococci (4.3%) (Broides *et al.,* 2009). Common anaerobic isolates are *Fusobacterium* and

Prevotella (Haraldsson *et al*, 2004). These are also the common normal flora of the nasopharynx. However, microbial investigations of chronic otitis media reveal other bacteria as the causative agent. The bacteria may be aerobic like *Ps. aeruginosa, Escherichia coli, Staph. aureus, Strep. pyogenes, Pr. mirabilis, Klebsiella* species or anaerobic like *Fusobacteium, Bacteroides, Peptostreptococcus, Proprionibacterium*, etc.

Fungal infection accounts for about 15.5% to 24.8% of chronic otitis media cases. (Talwar *et.al*, 1988). *Aspergillus* species especially *A. niger, Candida* species *Penicillium* species are found to be responsible for such infections (Talwar *et al*, 1988).

Different studies have shown that a single organism or multiple organisms may cause the infection. In case of bilateral infection, the organism isolated may be the same for both ear or different for different ear (Senior *et al.*, 1984).

Several researches conducted in Nepal have shown frequent isolation of *Ps. aeruginosa* and *Staph. aureus* from the ear discharge of patient suffering from CSOM (Shrestha *et al*, 2011; Sanjana *et al*, 2011) Organisms isolated in lower number include *Klebsiella* species, *Proteus* species, *E. coli, Citrobacter* species etc.

2.3 Pathogenesis

The normal flora of the nasopharynx generally causes the disease when they get entry to the mucosal lining of the middle ear. The normal flora of external ear of human includes bacteria and fungi (Forbes *et al* 2007). Different staphylococci, diphtheroids, streptococci, *Alloiococcusotitis* are the major bacteria isolated from external ear canal and cerumen (Stonman *et al*, 2001). Similarly, *Propionibacterium acnes*, enterobacteriaceae, *Ps. aeruginosa* are also found as the normal flora of human external ear (Forbes *et al* 2007). The middle and inner ear is usually sterile.

The understanding of the anatomy of human ear helps to understand the pathogenesis of the otitis media. Human ear can be divided into three anatomic parts: external, middle and inner parts. The external ear consists of pinna and auditory canal. It collects the sound from the environment and transmits it to the middle ear. The middle ear consists of tympanic membrane and auditory ossicles. They transmit the vibration transmitting in the air medium along the external ear to the inner ear, through solid medium. The inner ear consists of semicircular canals, cochlea and nerve fibres. The sound is transmitted in the liquid medium through the cochlea to the brain via cochlear nerves. Semicircular canals of inner ear are the organs of balancing. The middle ear is continuous with the respiratory system via eustachian tube (Noback *et al.* 1995; Forbes *et al*, 2007)

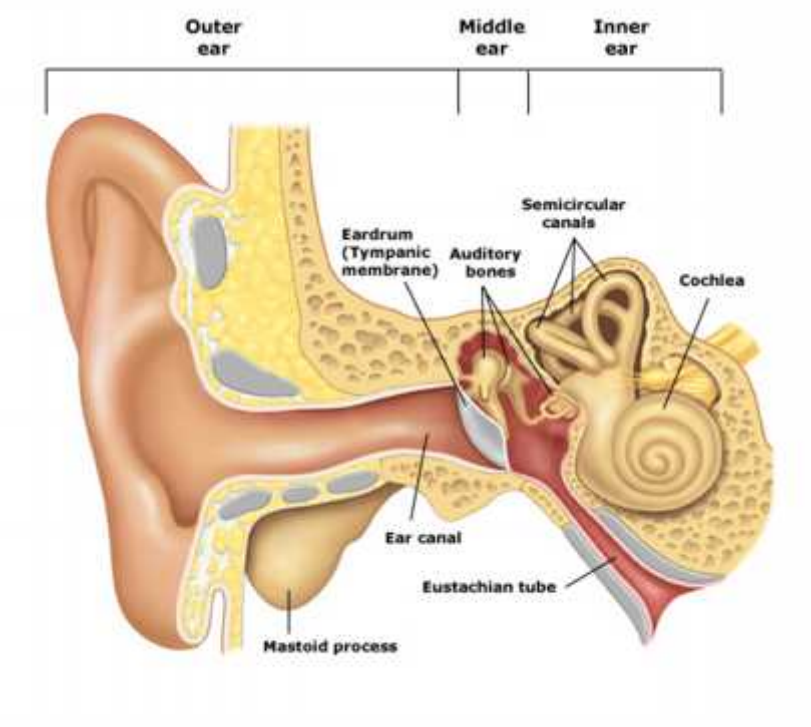

Figure 1: Anatomy of human ear.

Several factors play important role in preventing the entry and colonization of the middle ear by these organisms. Lee H *et al.* (2004) demonstrated that lysozyme and β defensins secreted by respiratory mucosal epithelia, including those of the middle ear and eustachian tube, can inhibit the organisms causing otitis media. The organisms they tested included nontypeable *H. influenzae*, *M. catarrhalis*, and *Strep. pneumoniae*. Similarly, Sabharwal V *et al* (2009), used animal model in their study and concluded that limiting complement C3 protein deposition on the surface of *Strep. pneumoniae* correlates with increased incidence of otitis media after nasopharyngeal colonization and barotrauma in the animal model.

The disease is considered as multifactorial as various factors can lead to the development of disease (Bakaletz, 2010). High rates of otitis media have been attributed to viral and bacterial infection of upper respiratory tract; eustachian tube dysfunction; young age and immature or impaired immunologic status; familial predisposition; presence of older siblings; male sex; bottle feeding; day-care attendance; passive smoking; overcrowding; poor hygiene; high colonization of naso-pharynx with potential pathogens; etc (WHO/CIBA, 1996). Frequently, the infection of upper respiratory tract leads to the inflammation of the middle ear, as it is also a part of the upper respiratory tract (Alberti, n.d). Usually the viral infection of the upper respiratory tract results in the clogging of the respiratory mucosa resulting in the blockage of the Eustachian tube. This creates the negative air pressure in the middle ear and the potential pathogens are aspirated into the middle ear where suppurative effusion accumulates (WHO/CIBA, 1996).

Chronic infection results due to continuing negative pressure within the middle ear, which traps the effusion in the ear and secondary infection, may be caused due to reflux of pathogens from respiratory tract or direct entry of organisms like *Ps. aeruginosa*, *Staph. aureus*, etc from the external ear canal (WHO/CIBA, 1996).

Otits media is seen as sequelae to many viral and bacterial diseases as well, such as scarlet fever, meseales, influenza, pneumonia, etc. Acute otitis media (AOM) is

a common complication of upper respiratory tract infection of both viral and bacterial etiology. (Pettigrew *et al.*, 2011).

2.4 Risk factors

Different environmental and host factors may contribute in the development of CSOM. Daycare attendance, overcrowding, passive smoking, bottle feeding, presence of older siblings, seasonal variations, genetics, immunodeficiency, birth defects like cleft palate or Down's syndrome (WHO/CIBA, 1996), lower socioeconomic status and treatment with tympanostomy tubes (Vander Veen *et al*, 2006), air pollution (Zemek, 2010), use of pacifier (Rovers *et al.*, 2008) are some of the predisposing factors for otitis media.

One of the important risk factors of otitis media is attendance at day care center. The risk of infection increases with the number of close contacts with other infected children and these contacts increase in day care centers, increasing the risk of a child acquiring the infection (Uhari *et al*, 1996). Similarly, large family size and overcrowding also increases the risk of acquiring the infection (WHO/CIBA, 1996).

Economic status of the family is also a predictor of otitis media. Ear infection is more frequent in children from lower socio-, economic background than the privileged children (Chandha *et al.*, 2006; Lasisi *et al.*, 2007).

Passive smoking causes inflammation of the mucosa of middle ear cleft and also reduces mucociliary clearance, which consequently increases risk of infection. Similarly, air pollution has been found to have direct correlation with emergency hospital visits for otitis media (Zemek, 2010).

Genetics play an important role in the etiology of CSOM. Males have a higher incidence of CSOM than females, history of infection in siblings and parents are related to a child's risk of developing CSOM (WHO/CIBA, 1996). Studies have shown that some aboriginal communities like Canadian Eskimos, Native

Americans, Alaskans and Australian Aboriginals have higher incidence of otitis media than in other group of people (Maxson *et al.*, 1996).

Other predictors of middle ear infection are previous tympanostomy tube insertion, more than three upper respiratory tract infections in 6 months time, low socio-economic status and education level of parents (Vander Veen *et al*, 2006).

Bottle feeding at bed increases the risk of otitis media (WHO/CIBA, 1996), while breast feeding for at least first three months of life decreases the risk of otitis media in first year of life (Maxson *et al.*, 1996). Similarly, use of pacifier has also been found to be the risk factor for recurrent AOM (Rovers *et al,* 2008)

Several studies have revealed that having older siblings is a prognostic factor for otitis media (Rovers *et al.,* 1999; Vander Veen *et al.*, 2006). Similarly, people with cleft palate, cleft uvula, submucosal cleft, eustachian tube dysfunction and immune deficiency state like chronic granulomatous diseases, immunoglobulin deficiencies, malignancies, Human Immuno-deficiency Virus infection are more prone to developing ear infection (Maxson *et al.*, 1996).

2.5 Epidemiology

About 28,000 deaths due to otitis media were recorded all over the world and largely among developing countries in 1990 (Child and Adolescent Health and Development Prevention of Blindness and Deafness World Health Organization, 2004).

The 1996 World Development Report estimated that about 2.163 million DALYs were lost from otitis media, 94% of which came from the developing world. WHO had reported that South-East Asia and the Western Africa had both the highest prevalence of CSOM and the highest number of deaths and DALYs from otitis media. Otitis media cases in these two regions accounted for 61% of total deaths and 57% of total DALYs (WHO/CIBA, 1996).

The prevalence of chronic otitis media ranges from 1-46% around the world. A prevalence of ≥4% of chronic otitis media is considered a massive health problem and immediate attention in targeted populations is required (WHO/CIBA, 1996). There have been recent comparisons of the burden of mortality and loss of DALY between otitis media and other diseases of importance (WHO/CIBA, 1996). The studies show that the burden from otitis media is greater than from trachoma, and comparable with that of polio (WHO/CIBA, 1996).

Recent study by Monasta *et al* (2012) reported the global incidence rate of AOM to be 10.85% i.e. 709 million cases each year with 51% of these occurring in under-fives. CSOM incidence rate was reported to be 4.76% i.e. 31 million cases, with 22.6% of cases occurring annually in under-fives.

Otitis media-related hearing impairment had a prevalence of 30.82 per ten-thousand. Each year 21 thousand people died due to complications of otitis media. For South Asia, they estimated the incidence of AOM to be 14.52 (minimum 13.84 and maximum 15.21) and incidence of CSOM to be 6.56 with minimum 6.05 and maximum 7.08.

A pilot study in Nepal has showed that approximately 16% population above 5 years of age suffers from otitis media and more than 55% of these cases occur in school going children, most of them belonging to the lower socio-economic class (Maharjan *et al*, 2006). Another study by Adhikari *et al* (2008) showed that among the otological diseases, CSOM is the second most common problem after wax. Wax was found in 60.6% children and CSOM in 5.7% cases. Other ear problems that followed were OME (3.7%), AOM (1.4%). Adhikari *et al* in 2007 demonstrated the prevalence of CSOM in children of Kathmandu to be 5.4%. Similarly, Adhikari *et al.* (2009) reported the prevalence of CSOM in urban children to be 5.0%.

2.6 Clinical features

Otoscopic examination of infected ear may reveal perforated or congested tympanic membrane with presence of fluid behind the ear drum or in the ear canal (WHO/CIBA, 1996). Otorrhoea, in CSOM, is mucoid to purulent, painless and without fever. However, fever may develop if the case is accompanied by otitis externa or complicated by an extracranial or intracranial infection (WHO, 2004). AOM caused by *Strep. pneumoniae* is severe with high fever, otalgia and more frequent redness and bulging of tympanic membrane (Palmu *et al*, 2004) *H. influenzae* is frequently associated with recurrent AOM. Group A streptococcus causes severe infection in older children and is frequent cause of spontaneous perforation and mastoitidis. AOM caused by *M. catarrhalis* is more common at younger age and is characterized by a higher proportion of mixed infections (Broides *et al.,* 2009).

2.7 Management of the infection

Watchful waiting of AOM in patients over the age of six months, who have mild otalgia and fever less than 39°C, is the recommended management strategy as AOM can resolve spontaneously but for children below 6 months and for the patients with severe otalgia and high fever, amoxycillin is recommended to minimize complications (McWilliams *et al*., 2011).

For the cases of CSOM with mucoid, foul- or non-foul smelling, occasionally profuse otorrhoea oozing through a central tympanic perforation involving neither the drum margin nor its posterior region (tubo-tympanic CSOM), antibiotics are administered to control infection and eliminate discharge. However, in the infection involving the hidden upper recesses (attic) of the middle ear and the mastoid antrum (attico-antral disease), antibiotics are useless as the site of infection is too deep to be reached by the antibiotics (WHO, 2004).

Increasing rate of MRSA isolation from AOM cases has prompted many researches to find out appropriate method of treatment. Al-Shawwa *et al.* (2005),

in their study, concluded that Trimethoprim-sulfamethoxazole combined with a topical agent (gentamicin sulfate or polymyxin B sulfate–neomycin sulfate–hydrocortisone) is a good alternative for initial, empirical therapy for AOM with otorrhea if MRSA is suspected.

Tympanoplasty of chronic dry perforation is the surgical management of the chronic ear infection (WHO/CIBA, 1996).

2.8 Antibiotic susceptibility test

Bacteria may show intrinsic resistance or acquired resistance to any antibiotic. Among the two, acquired resistance is of great concern to medical personnel. Bacteria may acquire resistance to any drug either by mutation or through exchange of genetic material among the same or closely related species. The sudden acquisition of resistance to antibiotics poses difficulties in treating infections. Moreover, rise of Multi-Drug Resistant (MDR) strains have posed greater threats to the health personnel. It is, therefore, better to determine the antibiotic susceptibility pattern of the causative organism before the administration of the drug.

Disc diffusion method for determination of antibiotic susceptibility of microorganisms is a rapid method. Kirby-Bauer disc diffusion test is a standardized procedure for single antimicrobial disc susceptibility testing set by WHO in 1961. Currently, the Clinical Laboratory Standards Institute (CLSI) is responsible for updating and modifying the original procedure of Kirby and Bauer through a global consensus process. In this method, bacteria are inoculated in Mueller-Hinton agar, along with antibacterial discs. After proper incubation, zone of inhibition is measured and results interpreted.

Resistance pattern of any isolate is ever-changing and it also differs from one geographic region to other. The composite susceptibility of *Strep. pneumoniae, H. influenzae* and *M. catarrhalis* to amoxycillin was found to be 76% and corresponding susceptibility to amoxycillin-clavulanate to be 94% (Jacobs *et al*,

1998). In their study, 31% of *H. influenzae* and 100% of *M. catarrhalis* produced β-lactamase. 30% of *Strep. pneumoniae*, they isolated, were intermediately or fully resistant to penicillin. Susceptibility of *Strep. pneumoniae* to non β-lactam antibiotics ranged from 59% for trimethoprim-sulfamethoxazole to 90% for chloramphenicol. The prevalence of resistant *Strep. pneumoniae* decreased with increase in age (Jacobs *et al*, 1998).

Majority of isolated *Ps. aeruginosa*, *Staph. aureus, Klebsiella* species and *Proteus* species from adult population were susceptible to ceftazidime, azithromycin, ceftriaxone, cefuroxime and gentamicin (Oni *et al*, 2002).

Among the *Staph. aureus, Ps. aeruginosa* and *Pr. mirabilis. E. coli, Acinetobacter* species and streptocci isolated from CSOM cases, 58.3% of the isolates were susceptible to ciprofloxacin and 39.7% were resistant to it. Similarly, 73.1% of isolates were sensitive to gentamicin and 21.2% resistant to it. Likewise, susceptiblity to trimethoprim-sulphamethoxazole was seen in 22.4% of isolates and resistance in 42.3% (Lodhi *et al,* 2010).

Among the *Ps. aeruginosa* isolated from CSOM cases, 43.6% were susceptible to 10 antibiotics tested: aminoglycosides, cephalosporins, anti-pseudomonal penicillin, imipenem and quinolones; and 53.7% were resistant to more than one antibiotic (Lee *et al*, 2012).

The common causative agents of otitis media isolated in Nepal are *Staph. aureus*, *Ps. aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, *Citrobacter* species, *Proteus* species, etc. (Jha *et al*, 2007; Sanjana e*t al*, 2011). Most of the *Ps. aeruginosa* are susceptible to tobramycin (93.2%) and ceftazidime (91.5%). Similarly, *Staph. aureus* isolates are susceptible to cloxacillin (95.2%) and gentamicin (83.3%). Only 7.1% are sensitive to ampicillin and 26.1% to ciprofloxacin (Sanjana e*t al,* 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

A complete list of materials, equipments, chemicals, reagents, antibiotics and media used for this study are given in the appendix B.

3.2 Methods

Cross sectional study was carried out in the Microbiology Laboratory of Shree Birendra Hospital, Chhauni from January 2012 to July 2012. Ethical approval for the conduction of the study was obtained from the hospital authority. Our study population was the clinically diagnosed cases of AOM and CSOM, visiting the ENT OPD of the hospital. The aural discharges from 153 patients were collected in the sterile micro-swabs and processed in the laboratory to determine the bacteriology of the discharge and find the antibiotic susceptibility pattern of the isolates.

3.3 Collection of sample

Sterile micro-cotton swabs were used to collect the pus sample from the affected ear(s) of the patients with aural discharge. Patients were diagnosed for AOM and CSOM by the ENT specialists of Shree Birendra Hospital, Chhauni and the swabs were taken by the specialists themselves.

3.3.1 Preparation of micro-cotton swab stick

A piece of cotton was rolled on the tip of a bamboo stick firmly. The swab thus prepared was then placed in the test tube. The test tube was cotton plugged tightly. The test tube was then autoclaved at 121˚C and 15 lbs for 15 minutes.

3.3.2 Sample collection

Actively discharging cases of otitis media were diagnosed by ENT specialists. A sterile aural speculum was introduced into affected ear. The sterile micro-cotton swab was then inserted through the speculum without touching the speculum. The pus was collected with full aseptic precautions by the specialist. The micro swab was placed back into the sterile test tube. The tubes were labeled properly. Questionnaire was filled for the patients whose aural discharge was obtained.

3.4 Sample transportation

The samples, thus collected, were transported to the microbiology lab within 2 hours.

3.5 Sample processing

The swabs were brought to the laboratory. The swabs were streaked onto the blood agar, chocolate agar and Mac Conkey agar plates. Smears were prepared from the swab and Gram staining was performed. Blood agar plate and Mac Conkey agar plates were incubated at 37˚C for 24 to 48 hours. Chocolate agar plate was placed in the candle extinction jar and incubated at 37˚C for 24 to 48 hours. After incubation, the plates were observed for the growth of the bacteria. The colony characteristics of the isolated bacteria were noted. Gram staining of the isolated bacterial colonies was performed. Identification of the isolates was done on the basis of their morphology and biochemical tests. Antibiotic sensitivity test was carried out according to Kirby Bauer Disc Diffusion Method.

3.6 Identification of isolates

For the identification of isolates, the isolated colonies of the organism were further sub cultured in the nutrient agar. The isolated colonies from the nutrient agar were Gram stained and then inoculated into different biochemical media for identification.

3.6.1 Biochemical tests for Gram positive organisms

After observation under oil immersion, Gram positive cocci in cluster and Gram positive cocci in chains were further processed differently.

3.6.1.1 Gram positive cocci in cluster

Golden yellow colored colonies from nutrient agar, which were Gram positive cocci in cluster, were suspected to be *Staph. aureus*. Catalase and oxidase tests of the isolates were performed. Isolated colonies were picked and slide coagulase test was performed. For confirmation, tube coagulase test was performed for those isolates that gave positive slide coagulase test.

3.6.1.2 Gram positive cocci in chains

Gram positive cocci in chain or pairs producing haemolysis on blood agar were suspected to be *Streptococcus* species. Catalase and oxidase tests of the isolates were performed. The organisms were identified after examining the zone of inhibition around the bacitracin or optochin disc placed at the site of primary inoculation on blood agar.

3.6.2 Biochemical tests for Gram negative organism

Colony characteristics on MA and NA were observed. Catalase and oxidase tests of the isolates were performed. The Gram negative isolates were inoculated into various biochemical media. TSI agar slant, SIM, Citrate and Urease media were used. The inoculated media were incubated at 37˚ C for 18-24 hours. The biochemical media were observed for change in color after adding appropriate reagents. The organisms were identified.

3.6.3 Identification of *Haemophilus* spp

Gram negative coccobacilli which produced seminal odour in chocolate agar culture were suspected of *Haemophilus* species. The suspected colony was

transferred into the sterile saline to make a suspension equivalent to 0.5 MacFarland. The suspension was then spread over the nutrient agar with the help of a sterile cotton swab. X, V and XV factor discs were placed at least 4-5 cm apart from each other on the agar surface. The plate was incubated overnight at 37˚ C in ambient air. After incubation, the plates were observed for growth of organisms around the discs.

3.7 Antibiotic susceptibility test

After identification of the organisms, 0.5 MacFarland equivalent of suspension of the organism was prepared. The suspension was swabbed on the MHA agar to produce a lawn culture of the organism. For *Strep. pyogenes*, blood agar plate and for *H. influenzae*, chocolate agar plate were used. The plate was left undisturbed for few minutes to allow the suspension to be absorbed by the agar. The appropriate antibiotic discs were placed on the swabbed agar plate. Each disc was pressed down to ensure complete contact with the agar surface. The discs were kept at least 24 mm apart from each other. The plate was inverted and incubated at 37°C. After 16 to 18 hours of incubation, each plate was taken out. The diameters of the zones of complete inhibition were measured. While testing for vancomycin sensitivity on *Staphylococcus*, the plates were examined after 24 hours of incubation. The sizes of the zones of inhibition were interpreted by referring to standard chart and the organisms were classified as sensitive, intermediate or resistant to specific antibiotic.

3.8 Purity plate

Purity plate culture of each batch of biochemical test was performed to observe whether the tests were conducted in aseptic condition or not. It was performed during each biochemical tests. Half of the NA was inoculated before the test and half after the biochemical test was performed. This plate was incubated at 37˚C for 24 hours. The growth of same organism in pure form in both pre and post inoculation showed the maintenance of aseptic condition.

3.9 Quality control

Quality of each test was maintained by using standard procedures. The quality of each agar plates and biochemical media prepared was tested by incubating one plate and one set of biochemical media of each lot without inoculating any organisms. For stains and reagents, whenever a new batch of them were prepared, a control smear was stained to ensure correct staining reaction.

Similarly, to check the quality of swab sticks prepared, few swab sticks from each batch were streaked on the MA, BA and CA and the plates were incubated at 37˚C for 24 hours. The absence of growth in all the plates showed the maintenance of sterile condition.

Quality of sensitivity tests was maintained by maintaining the thickness of MHA at 4 mm and the pH at 7.2-7.4. Similarly antibiotic discs containing the correct amount as indicated and within their expiry date were used.

Strict aseptic conditions were maintained while carrying out all the procedures.

3.10 Data management and analysis

Data were recorded into the notebook after the daily work and then to the computer.

SPSS 16 and MS excel 2007 were used for data analysis and generation of the result. Chi square test was performed to determine the p-value of the result obtained.

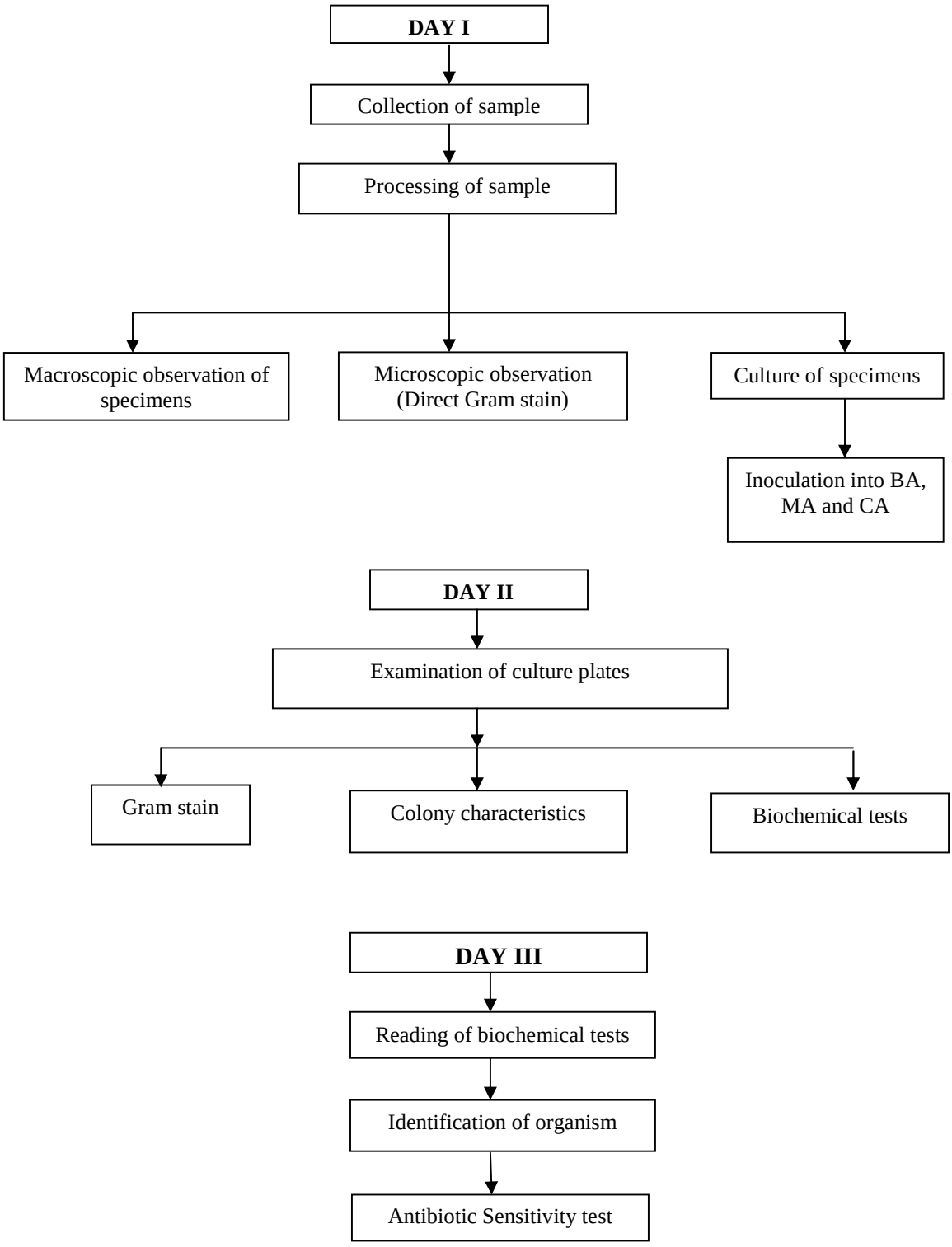

Figure 2: Flow chart of laboratory work

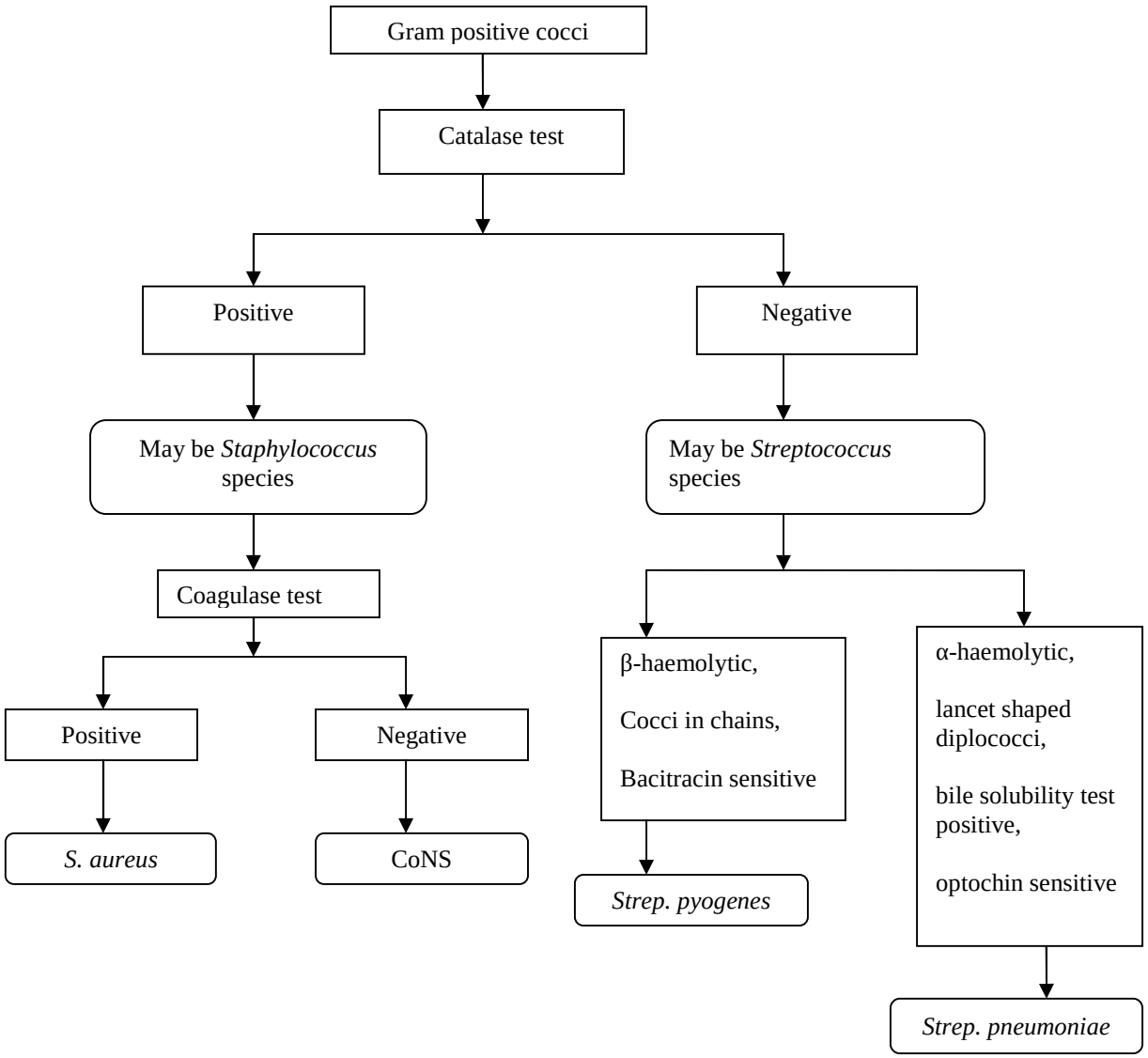

Figure 3: Identification of Gram positive cocci.

CHAPTER IV

RESULTS

4.1 Pattern of growth

From the 153 samples collected, 128 (84.31%) samples showed bacterial growth, while in 25 (15.69%) samples there were no bacterial growth (Figure 5). The samples which gave bacterial growth were considered growth positive samples while the samples producing no growth or fungal growth only were reported as growth negative samples.

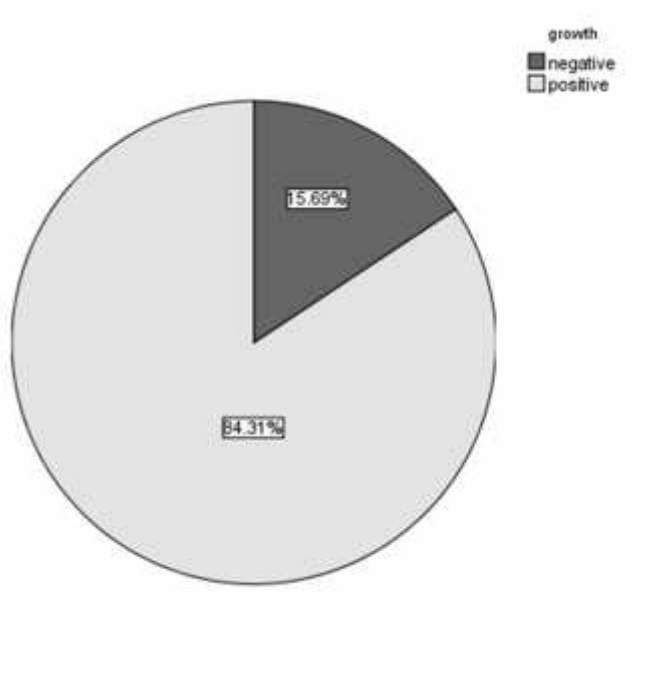

Figure 5: Pie Chart showing growth pattern.

4.2. Gender-wise distribution of sample

In our study, 58 samples were from women and 95 were from male. Eighty three samples from males (87.39%) and forty five samples from females (77.58%) gave bacterial growth (Table 1). Our study showed the higher number of male cases of otitis media than female cases.

Table 1: Distribution of cases according to sex

Sex	Number	Positive Sample	Percent of Positive Sample
Male	95	83	87.39
Female	58	45	77.58

4.3 Pattern of pure culture and mixed growth

Of the 128 positive samples, pure culture was obtained from 119 (93%) samples and mixed growth from 9 (7.0%) samples (Table 2). All the nine samples yielding mixed growth had two types of bacteria in different combination. Hence, from 119 pure cultures and 9 mixed cultures, the total of 136 different bacterial isolates was obtained.

Table 2: Frequency of pure culture and polymicrobial growth

	Number	Percent
Pure culture	119	93
Polymicrobial growth	9	7
Total	128	100

Comparison of single or polymicrobial isolation of bacteria between male and female cases is presented in figure 6. Single organism isolation was proportionally higher in male than in female while polymicrobial infection was observed in greater number of female than male (Figure 6).

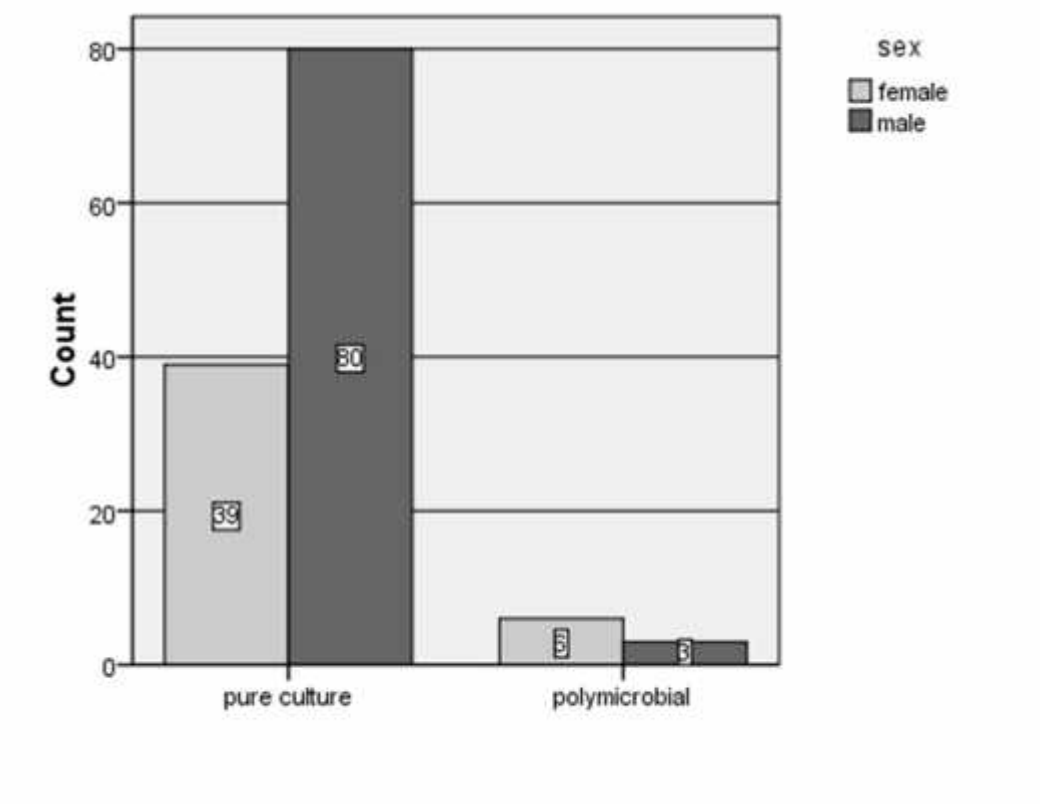

Figure 6: Distribution of pure culture and polymicrobial culture between the sexes

4.4 Age-wise distribution of the sample

The youngest patient included in our study was of 1 year and the oldest was a 75 years old male. The maximum number of cases (56 out of 153) was seen in the age group 20-30. The number of male cases was greater in all age groups except in age groups 30-40 and 50-60 (Table 3). The p value for the age-wise distribution of the growth of bacteria in this study was 0.593 (>0.05) so difference in number of isolates in different age group is statistically insignificant.

Table 3: Distribution of cases according to age

Age Group	Sex		Total	No. of Growth Positive Sample	P Value
	Male	Female			
0-10	14	10	24	20	>0.05
10-20	17	11	28	25	
20-30	42	14	57	48	
30-40	6	8	14	9	
40-50	8	5	13	11	
50-60	3	9	11	10	
60-70	2	0	2	2	
70-80	3	1	4	3	
Total	95	58	153	128	

4.5 Bacteriological analysis

4.5.1 Distribution of gram positive and negative isolates

From one hundred and nineteen pure cultures and nine mixed cultures, a total of one hundred and thirty six isolates were obtained. The total of eighty seven isolates was Gram positive and forty nine isolates were Gram negative (Table 4). However, wider varieties of Gram negative bacteria were isolated than Gram positive bacteria (Table 6). The frequency of Gram positive organisms was higher in both male and female cases (Table 5). The p value (>0.05) shows that the distribution of organisms between the two sexes seen in our study is not significant.

Table 4: Distribution of Gram positive and negative organisms

Organism	**Number**	**Percent**
Gram positive	87	64
Gram negative	49	36
Total	136	100

Table 5: Distribution of Gram positive and Gram negative organism according to sex

	Gram positive	**Gram negative**	**Total**	**P value**
Female	29	21	50	>0.05
Male	58	28	86	
Total	87	49	136	

4.5.2 Pattern of bacterial isolates

The most common bacterial isolate obtained was *Staph. aureus. Staph. aureus* was isolated from 70 (51.5%) cases followed by *Ps. aeruginosa* from 27 (19.9%) cases. Other organisms isolated were Coagulase Negative Staphylococci (CoNS), *Klebsiella* species, *E. coli, C. fruendii, Pr. mirabilis, Strep. pyogenes* and *H. influenzae*. The distribution of those organisms between male and female cases is demonstrated in table 6.

Table 6: Distribution of organisms according to sex

Organisms	**Sex**		**Total**	**Percent**
	Female	**Male**		
Staphylococcus aureus	22	48	70	51.5
Pseudomonas aeruginosa	13	14	27	19.9
CONS	7	9	16	11.8
Escherichia coli	1	6	7	5.1
Klebsiella pneumoniae	5	1	6	4.4
Klebsiella oxytoca	2	1	3	2.2
Citrobacter fruendii	0	4	4	2.9
Proteus mirabilis	0	1	1	0.7
Streptococcus pyogenes	0	1	1	0.7
Haemophilus influenzae	0	1	1	0.7
Total	50	86	136	100

4.5.3 Pattern of bacteria in mixed growth

Among the 9 samples yielding mixed growth, 4 produced the combination of *Staph. aureus* and *Ps. aeruginosa*. *Staph. aureus* and CoNS were isolated from 3 samples. Similarly, the mixed growth of *Ps. aeruginosa* and CoNS was obtained from one sample and the combination of *P. aeruginaosa* and *Klebsiella* spp was seen in one sample. *P aeruginosa* was found to be associated with all the three types of other bacteria isolated in mixed growth. (Table 7).

Table 7: Organisms isolated in mixed growth

Combination of organisms	**Number**
Staph. aureus and *Ps. aeruginosa*	4
Staph. aureus and CoNS	3
Ps. aeruginosa and CoNS	1
Ps. aeruginosa and *Klebsiella* species	1
Total	**9**

4.5.4 Pattern of bacteria isolated in different age-group

As the number of cases in the age–group 20-30 was the greatest, the number of bacterial isolates was also the greatest in this group. Fifty one out of 136 isolates were from this age group. The common isolate, *Staph. aureus* was isolated from all the age groups and the second most common isolate, *P. aeruginisa* was isolated from all the age groups except the age group 60-70. The least common organisms like *C. fruendii, K. oxytoca, Pr. mirabilis, Strep. pyogenes* were isolated from the patients younger than 30 years of age. (Table 8).

Table 8: Age-wise distribution of the isolates

Organism	**Age group**								**Total**
	0-10	**10-20**	**20-30**	**30-40**	**40-50**	**50-60**	**60-70**	**70-80**	
Staph. aureus	10	14	26	6	6	4	2	2	70
Ps. aeruginosa	2	4	13	2	3	2	0	1	27
CONS	4	3	4	1	2	1	0	1	16
E. coli	1	2	3	1	0	0	0	0	7
K. pneumoniae	1	1	1	0	0	3	0	0	6
K. oxytoca	2	1	0	0	0	0	0	0	3
C. fruendii	0	1	3	0	0	0	0	0	4
Pr. mirabilis	0	0	1	0	0	0	0	0	1
Strep. pyogenes	1	0	0	0	0	0	0	0	1
H. influenzae	0	0	0	0	0	1	0	0	1
Total	21	26	51	10	11	11	2	4	136

4.6 Antiobiotic susceptibility pattern of the isolates.

In our study, the isolated *Staph. aureus* were mostly susceptible to cefoxitin. 67.1% of the total *Staph. aureus* isolates were susceptible to cefoxitin. Erythromycin was the second drug most effective against the *Staph. aureus* isolates (58.6%). Similarly, 57.1% of the isolates were susceptible to co-trimoxazole and 50% of the isolates were susceptible to ciprofloxacin. The least effective drug was found to be amoxycillin (45.7%). (Table 9). All the cefoxitin resistant *Staph. aureus* were susceptible to vancomycin.

Table 9: Antiobiotic susceptibility pattern of *Staph. aureus*

Organism (No. of isolates)	**Antibiotics used**	**Susceptibility pattern**					
		Sensitive		**Intermediate**		**Resistant**	
		Number	**%**	**Number**	**%**	**Number**	**%**
Staph. aureus (70)	Amoxycillin	32	45.7	-	-	38	54.3
	Cefoxitin	47	67.1	-	-	23	32.9
	Co-Trimoxazole	40	57.1	18	25.7	12	17.1
	Ciprofloxacin	35	50.0	18	25.7	17	24.3
	Erythromycin	41	58.6	14	20	15	21.4

All the isolates of *Ps. aeruginosa* in our study were susceptible to imipenem. 92.3% of the *Ps. aeruginosa* isolates were susceptible to piperacillin. Ceftazidime and amikacin were effective against 88.9% of the total *Ps. aeruginosa* isolates. Among the antibiotics tested, ciprofloxacin was effective against the least percent of the isolates (74.1%). (Table 10).

Table 10: Antibiotic susceptibility pattern of *Ps. aeruginosa*

Organism (No. of isolates)	**Antibiotics used**	**Susceptibility pattern**					
		Sensitive		**Intermediate**		**Resistant**	
		Number	**%**	**Number**	**%**	**Number**	**%**
Ps. aeruginosa (27)	Ceftazidime	24	88.9	-	-	3	11.1
	Amikacin	24	88.9	1	3.7	2	7.4
	Piperacillin	25	92.3	-	-	2	7.4
	Ciprofloxacin	20	74.1	2	7.4	5	18.5
	Imipenem	27	100	-	-	-	-

Gentamicin was the most effective drug against *E. coli* isolates in our study. 71.4% of the isolates were susceptible to gentamicin. Similarly, 57.1% of the isolates showed susceptibility towards co-trimoxazole. However, cefotaxime and ciprofloxacin was effective against only 42.9% of the isolates. The least antibacterial activity against *E. coli* was shown by amoxycillin. Only 28.6% of the *E. coli* isolates were susceptible to amoxycillin. (Table 11).

Table 11: Antibiotic susceptibility pattern of *E. coli*

Organism (No. of isolates)	**Antibiotics used**	**Susceptibility pattern**					
		Sensitive		**Intermediate**		**Resistant**	
		Number	**%**	**Number**	**%**	**Number**	**%**
E. coli (7)	Amoxycillin	2	28.6	2	28.6	3	42.9
	Gentamicin	5	71.4	1	14.3	1	14.3
	Cefotaxime	3	42.9	1	14.3	3	42.9
	Ciprofloxacin	3	42.9	1	14.3	3	42.9
	Co-Trimoxazole	4	57.1	1	14.3	2	28.6

Of the 4 isolates of *C. fruendii*, all were susceptible to gentamicin and cefexime. Three of the isolates were sensitive to ciprofloxacin and co-trimoxazole. One isolate was resistant to ciprofloxacin and one isolate showed intermediate susceptibility to co-trimoxazole. However, only one *C. fruendii* isolate was found to be susceptible to amoxycillin. (Table 12).

Table 12: Antibiotic susceptibility pattern of *C. fruendii*

Organism (No. of isolates)	**Antibiotics used**	**Susceptibility pattern**					
		Sensitive		**Intermediate**		**Resistant**	
		Number	**%**	**Number**	**%**	**Number**	**%**
C. fruendii (4)	Amoxycillin	1	25	-	-	3	75
	Gentamicin	4	100	-	-	-	-
	Cefixime	4	100	-	-	-	-
	Ciprofloxacin	3	75	-	-	1	25
	Co- Trimoxazole	3	75	1	25	-	-

Among the four antibiotic discs used for *Klebsiella* species, gentamicin and ciprofloxacin were the most effective drugs. Seven (77.8%) of the nine *Klebsiella* isolates were susceptible to these antibiotics. Similarly, 6 (66.7%) *Klebsiella* isolates were susceptible to cefexime and co-trimoxazole. (Table 13).

Table 13: Antibiotic susceptibility pattern of *Klebsiella* species

Organism (No. of isolates)	**Antibiotics used**	**Susceptibility pattern**					
		Sensitive		**Intermediate**		**Resistant**	
		Number	**%**	**Number**	**%**	**Number**	**%**
Klebsiella species (9)	Gentamicin	7	77.8	1	11.1	1	11.1
	Cefixime	6	66.7	2	22.2	1	11.1
	Ciprofloxacin	7	77.8	-	-	2	22.2
	Co- Trimoxazole	6	66.7	1	11.1	2	22.2

All the isolated CoNS (16) were susceptible to cefoxitin. Amoxycillin was effective against 14 (87.5%) of the isolates. Similarly, 12 (75%) of the isolates were susceptible to erythromycin, 8 (50%) to ciprofloxacin and 7 (43.8%) to co-trimoxazole. (Table 14)

Table 14: Antibiotic susceptibility pattern of CoNS

Organism (No. of isolates)	**Antibiotics used**	**Susceptibility pattern**					
		Sensitive		**Intermediate**		**Resistant**	
		Number	**%**	**Number**	**%**	**Number**	**%**
CoNS (16)	Amoxycillin	14	87.5	-	-	2	12.5
	Cefoxitin	16	100	-	-	-	-
	Co-Trimoxazole	7	43.8	3	18.8	6	37.5
	Ciprofloxacin	8	50.0	5	31.2	3	18.8
	Erythromycin	12	75	1	6.2	3	18.8

Pr. mirabilis, *Strep. pyogenes* and *H. influenzae* were each isolated from three single cases. The antibiotic susceptibility test of those organisms showed that they were susceptible to all the antibiotics tested. *Strep. pyogenes* was tested against erythromycin, amoxycillin, cefotaxime and chloramohenicol. *Pr. mirabilis* was tested against amoxycillin, gentamicin, ciprofloxacin and co-trimoxazole. Similarly, *H. influenzae* was seen to be sensitive to cefotaxime, ciprofloxacin and azithromycin but resistant to amoxycillin and chloramphenicol (Table 15).

Table 15: Result of antibiotic susceptibility test for single isolates

Organism	Antibiotic	Susceptibility pattern
Strep. pyogenes	Erythromycin	Sensitive
	Amoxycillin	Sensitive
	Cefotaxime	Sensitive
	Chloramphenicol	Sensitive
Pr. mirabilis	Amoxycillin	Sensitive
	Gentamicin	Sensitive
	Ciprofloxacin	Sensitive
	Co-Trimoxazole	Sensitive
H. influenzae	Amoxycillin	Resistant
	Cefotaxime	Sensitive
	Ciprofloxacin	Sensitive
	Azithromycin	Sensitive
	Chloramphenicol	Resistant

CHAPTER V

DISCUSSION

Otitis media is the common ENT problem, affecting the people of all age group. Otitis media is a broad term, which includes many types of condition. However, our study included only those conditions which produced active ear discharge, like AOM and CSOM.

Otitis media is a multi-factorial infection which can be induced by different risk factors (Miller *et al*, 2010). Moreover, it can be caused by wide varieties of microorganisms. The aim of our study was to isolate the bacterial etiological agents of otitis media from the ear discharge and study their antibiotic susceptibility pattern.

Traditional swab method was used to collect the aural discharge from the patients. Though swab method is condemned for introducing contaminants, Raju *et al* in 1990 and Adoga *et al* in 2004 separately proved the reliability of the swab method in their studies. In their independent studies, they found that the swab method was as good as aspiration method and both produced similar results.

In our study, out of 153 samples, only 128 (84.31%) samples showed bacterial growth. Similar result was obtained by Oni *et al* (2002). In their study 82.4% of the samples showed positive culture. In another study, involving 1103 CSOM patients, Lee *et al* (2010) isolated bacteria from 81.4% of the sample. Since, our study was concerned at isolating the aerobic bacterial agents from ear discharge, fungal growth was reported as growth negative. The samples may not have produced growth in the cases of otitis media due to anaerobic bacteria or in the cases of viral etiology as well.

Literatures state that the prevalence of otitis media in male is higher than in female. Our study also revealed the similar pattern. Ninety five (62.1%) of the 153 samples were from male patients and the remaining 58 (37.9%) from female.

Our result agreed with the result of Lodhi *et al* (2010). In their study, 61.5% of the cases were males and 37.8% were females. Similarly, in the study by Adhikari *et al* (2009) 54% of the cases were males and 46% females. Though our study showed higher number of male cases than female cases, the infection rate (p-value=0.112) didn't differ significantly between male and female population. The higher incidence of infection in males than in females may be due to the more exposed life style of the males.

Of the one hundred and fifty three cases in our study, 119 (77.7%) samples yielded growth of single organism while two different organisms were isolated from 9 (5.8%) samples. The proportion of pure culture isolated in our study is similar to other such studies. Aslam *et al* in 2004 reported 76% pure culture and 23.9% of mixed growth from the ear discharge samples. Osazuwa *et al*, 2011 reported 72.6% single organism isolation and 3.51% mixed growth culture from the ear swab. Generally, in polymicrobial infection, one organism creates a suitable niche for the colonization and infection by another organism (Brogden *et al*, 2005). In our study, *Ps. aeruginosa* was found to be associated with all the other bacteria isolated in mixed growth (Table 7). It may be due to the active biofilm formation by *Ps. aeruginosa*, within which interaction of other bacteria becomes easy.

Age-wise distribution of the cases (Table 3) in our study showed that cases of otitis media were higher in the age group of 20-30. Though otitis media is commonly seen in children, the number of child cases in our study is smaller. This may be due to the fact that in our study, the samples were collected from ENT OPD only and not from pediatrics OPD. In the hospital, most of the pediatric cases of otitis media were solved by pediatricians and only the chronic cases or complicated cases were referred to ENT department. This resulted in the decreased number of child cases in our study. Results comparable to our study were obtained in the study by Kumar *et al.* (2011). In their study, out of 100 cases, 47 cases of 20-40 years of age gave positive growth and only 17 cases, below the age of 10, yielded positive growth.

In regard of Gram type of the isolated organisms, Gram positive bacteria accounted 64% of the total isolates while 36% of the isolates were Gram negative in our study. This result was opposite to that of Madana *et al* (2010). They isolated higher proportion of Gram negative bacteria, 58% of the total isolates were Gram negative. Kumar *et al.* (2011) also reported the predominance of the Gram negative bacilli (59.74%) in their study on CSOM. In our study, *Staph. aureus* was isolated in more than 50% of cases which resulted in the higher proportion of Gram positive bacteria than Gram negative bacteria.

Staph. aureus was the predominant organism isolated in our investigation. Seventy out of one hundred thirty six isolates (51.5%) were *Staph. aureus*. The second most common isolate was *Ps. aeruginosa* (19.9%). Besides, *E. coli* was reported from 5.1% samples, CoNS from 11.8%, *Klebsiella* species from 6.6% and *C. fruendii* from 2.9% of the samples. One isolates each of *Pr. mirabilis*, *Strep. pyogenes* and *H. influenzae* were also obtained.

Our results are in accordance with several other similar studies. Jha *et al* (2007) reported *Staph. aureus* as the most common bacterial pathogen isolated from ear discharge of patients visiting Om Hospital, Kathmandu. Shrestha *et al* (2011), in their study conducted among the patients visiting ENT OPD in Kathmandu University Hospital, Dhulikhel, also reported *Staph. aureus* as the common pathogen of otitis media followed by *Ps. aeruginosa*. In their study 32.2% of the isolates were *S. aurues,* 26.9% were *Ps. aeruginosa* and remaining other organisms. *Staph. aureus* was reported as the most common isolate (50.6%) from the ear discharge by Lodhi *et al* (2010), followed by *Ps. aeruginosa* (28.8%). Other isolates obtained in their study were *Pr. mirabilis*, *E. coli*, *Acinetobacter* and streptococci. Similarly, Srivastava *et al* (2010), also reported *Staph. aureus* as the major pathogen of CSOM, followed by *Ps. aeruginosa*. However, in contrast to our study, Sanjana *et al* (2011) isolated higher number of *Ps. aeruginosa* (35.1%) than *Staph. aureus* (22.0%) from the ear discharge samples obtained in College of Medical Sciences-Teaching Hospital, Bharatpur. Tahir *et al*, 2012 also

reported predominance of *Ps. aeruginosa* (45.6%) over *Staph. aureus* (25.6%) in the study conducted in Combined Military Hospital Rawalpindi, Pakistan.

Third most common isolate obtained in our study was CoNS (11.8%). CoNS were reported as third common isolate from ear discharge by Loy *et al* (2002) and Sanjana *et al* (2011) as well. CoNS are the predominant normal flora of the external ear (Stroman et al, 2001). The CoNS isolated in our study may be the contaminants from the ear canal. However, in the absence of isolation of other potential pathogens of otitis media, they may be the causative agent of the infection and their isolation couldn't be neglected.

Other less frequent organisms isolated in our study are enterobacteriaceae: *E. coli* (5.1%), *Klebsiella* species (6.6%), *C. fruendii* (2.9%) and *Pr. mirabilis* (0.7%); *Strep. pyogenes* (0.7%) and *H. influenzae* (0.7%). The pattern of isolation of organisms from enterobacteriaceae family in our study is comparable with that of other workers. *E. coli, Klebsiella* species, *Proteus* species were reported as the common etiological agent of otitis media after *Staph. aureus* and *Ps. aeruginosa* by Jha *et al* 2007, Lodhi *et al*, 2010, Sanjana *et al* 2011,Shrestha *et al* 2011, Tahir *et al*, 2012. Yildirim *et al* in 2005 studied the effect of temperature on the bacteriology of CSOM and concluded that isolation of enteric bacteria increases significantly with increase in the temperature of the climate. Since our study was conducted mostly during cooler months of the year, the frequency of these bacteria could have been less.

One isolates each of *H. influenzae* and *Strep. pyogenes* were recovered in our study. These two organisms are the common etiological agents of AOM (Segal *et al*, 2005). In our study, the number of AOM cases was very less so, the number of these organisms isolated is also very less.

Patient population, hygienic practices, variation in the climate, socio economic condition, etc affect the pattern of bacterial flora of otitis media. It is, thus, useful

to identify the etiological agent of the infection and determine their antibiotic susceptibility pattern for successful treatment of the case.

Knowledge of the local microbial pattern and their antibiotic susceptibility is essential for effective and cost-saving treatment. This will contribute to rational usage of antibiotics, success of treatment and avoidance of complications.

Sensitivity pattern of *Staph. aureus* (Table 9) in our study showed that 47 (67.1%) were sensitive to cefoxitin. Similarly, erythromycin was the second drug most effective against the *Staph. aureus* isolates (58.6%), co-trimoxazole was active against 57.1% of the isolates and 50% of the isolates were susceptible to ciprofloxacin. The least effective drug was found to be amoxycillin (45.7%) (Table 9). All the cefoxitin resistant *Staph. aureus* were susceptible to vancomycin.

In similar studies on *Staph. aureus* isolated from ear discharge, Jha *et al* (2007) reported 59.4% of *Staph. aureus* isolated to be sensitive to ciprofloxacin and 34.4% to be sensitive to erythromycin. In another study by Sanjana *et al* in 2011, 95.2% of the *Staph. aureus* isolated were susceptible to cloxacillin and 83.3% to erythromycin.

In our study, 32.9% of the isolated *Staph. aureus* were resistant to cefoxitin. *Staph. aureus* resistant to cefoxitin are reported as Methicillin Resistant *Staph. aureus* (MRSA). The report rate of MRSA from clinical samples is alarming. Kumari *et al* (2009) and Tiwari *et al* (2009) respectively reported 26.14% and 69.1% of the total *Staph. aureus* isolates to be MRSA in different tertiary care hospitals of Nepal. MRSA has been frequently isolated from the CSOM cases as well in various studies in Nepal as well as abroad. Sanajana *et al* (2011) reported 12% of the total *Staph. aureus* isolated from CSOM cases to be MRSA in Nepal. Taj *et al* in 2000 detected 37.9 % of *Staph. aureus* from CSOM patients to be MRSA and Iqbal *et al* in 2011 reported MRSA as 21.7% of the *Staph. aureus* isolates from the ear discharge in Pakistan. Similarly, a study conducted in Korea

by Park *et al* (2008) identified 24.8% of the total isolates from CSOM to be MRSA and in another study by Lee *et al* (2010) in Korea, 25.8% of such isolates were MRSA.

According to Suzuki *et al*, 2003, MRSA are more commonly isolated from otitis media cases than from any other otolaryngeal infection. Genetic analysis of MRSA from CSOM cases in Korea suggested that most of the MRSA isolated from the ear discharge are healthcare associated or hospital acquired (Yang *et al*, 2008).

In our study, all the twenty seven *Ps. aeruginosa* isolates were susceptible to imipenem while only 92.3% of them were susceptible to piperacillin. 88.9% of them were susceptible to ceftazidime and amikacin. Ciprofloxacin was the least active drug among the five antibiotics used. Only 74.1% of *Ps. aeruginosa* isolates showed sensitivity towards ciprofloxacin. (Table 10).

As compared to our results, Sanjana *et al* (2011) also reported that 91.5% isolates were sensitive to ceftazidime and 77.9% to amikacin. *Ps. aeruginosa* isolated in their study showed lower susceptibility to ciprofloxacin (50.8%) as compared to our study. In contrast, 95.8% of *Ps. aeruginosa* isolates in the study by Aslam *et al* (2004) showed susceptibility to ciprofloxacin. 83.3% of them were susceptible to amikacin. In the study by Mansoor *et al* (2009), sensitivity pattern of *Ps. aeruginosa* from CSOM showed that amikacin was active against 96% of isolates followed by ceftazidime 89%, ciprofloxacin 85%, imipenem 76%.

Lee *et al* (2010) studied the antibiotic susceptibility pattern of *Ps. aeruginosa* from CSOM cases in Korea and reported that such isolates were highly susceptible to imipenem, ceftazidime, amikacin and piperacillin/tazobactem. Our result also showed similar pattern.

All the CoNS isolated in our study were sensitive to cefoxitin. Amoxycillin was active against 87.5% of the isolates. Erythromycin showed activity against 75% CoNS isolated. Similarly, 50% of the isolates were susceptible to ciprofloxacin

and only 43.8% were susceptible to co-trimoxazole (Table 14). In the study by Loy AHC *et al* (2002), the CoNS isolated from the ear discharge showed sensitivity to clindamycin, erythromycin and cloxacillin.

Five out of seven *E. coli* isolates (71.4%), in our study, were sensitive to gentamicin, 57.1% with co-trimoxazole and 42.9% with cefotaxime and ciprofloxacin each. Only 2 (28.6%) of *E. coli* isolates were susceptible to amoxycillin. (Table 11). In similar studies by various workers, the patterns of sensitivity to different antibiotics were different. In 2002, Nwabuisi and Ologe reported 96.8% of the *E. coli* isolates to be sensitive to gentamicin, 56.3% to co-trimoxazole and only 43.8% to ampicillin. Similarly, Alsaimary *et al* (2010) reported that ciprofloxacin was active against 80% of the *E. coli* isolates, gentamicin against 66.6% and ampicillin against only 40% of *E. coli* isolates. A study by Iqbal *et al* (2011) suggested that 50% of the *E. coli* were susceptible to gentamicin and only 8.3% to ciprofloxacin.

Among *Citrobacter* isolates (Table 12), all the four isolates were susceptible to gentamicin and cefixime while ciprofloxacin and co-trimoxazole were active against three isolates. Only one isolate was susceptible to amoxycillin. In the study by Jha *et al* (2007) in Om Hospital, Kathmandu, 50% of the *C. fruendii* isolates were sensitive to ciprofloxacin. Contrary to our result, Iqbal *et al* (2011) reported greater activity of ciprofloxacin (83.3%) to gentamicin (66.5%). Similarly, only 25% of *Citrobacter* isolates were susceptible to gentamicin and not a single *Citrobacter* isolate was susceptible to amoxycillin in the study by Osazuwa *et al* (2011).

In our study, the susceptibility pattern of *Klebsiella* species (9) showed that 77.8% were susceptible to ciprofloxacin and gentamicin each and 66.7% to cefexime and co-trimoxazole each (Table 13). However, Jha *et al* (2007) reported that only 47.9% of the *Klebsiella* isolates were susceptible to gentamicin and 37.9% to ciprofloxacin. Similarly, Alsaimary *et al* (2010) stated that ciprofloxacin was

active against 66.66%, gentamicin against 53.33% and trimethoprim against 26.66% of the *Klebsiella* species.

Only one *Proteus* isolate was obtained in our study and it was susceptible to all the antibiotics used: amoxycillin, gentamicin, ciprofloxacin and co-trimoxazole. In the study conducted in Lahore, Pakistan, among *Pr. mirabilis* isolates 70.5% were sensitive to gentamicin, 64.7% were sensitive to ciprofloxacin and 64.7 were sensitive to both ciprofloxacin and gentamicin (Lodhi *et al*, 2010). Similarly, Srivastava *et al* (2010), in India, reported that ampicillin was active against 17%, macrolides against 34%, aminoglycosides against 67% and flouroquinolones against 90% of the *Proteus* species isolated from the CSOM samples.

In our study, *Strep. pyogenes* was susceptible to erythromycin, amoxycillin, cefotaxime and chloramphenicol. Similarly, *H. influenzae* was susceptible to cefotaxime, ciprofloxacin and azithromycin but resistant to amoxycillin and chloramphenicol. Alsaimary *et al* (2010), in their study reported that *Strep. pyogenes* was mostly sensitive to ciprofloxacin (80%) followed by gentamicin (66.66%) and ampicillin (53.33%). Similarly, in their study, 75% of *H.. influenzae* isolates were susceptible to ciprofloxacin, 30% to ampicillin and 50% to erythromycin.

Jacob *et al* (1998) studied the antimicrobial-resisitant pathogens in the ear discharge from seven different countries and found that 31% of the *H. influenzae* produced β lactamase. The composite susceptibility to amoxicillin for *Strep. pneumoniae, H. influenzae,* and *M. catarrhalis* was 76% and to amoxicillin-clavulanate was 94%.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Among the 153 samples collected from the patients suffering from otitis media and those visiting ENT OPD of the hospital, 128 (84.31%) of the samples yielded bacterial growth out of which 119 (93%) produced single bacteria and remaining 9 (7%) produced mixed culture of two different bacteria. Males had greater rate of infection and the highest number of cases was observed in the age group of 20-30. 64% of the total isolates were Gram positive and only 36% were Gram negative. All together, nine different types of bacteria were isolated. The bacterial isolates in decreasing order of occurrence are *Staph. aureus* (51.5%), *Ps. aeruginosa* (19.9%), CoNS (11.8%), *Klebsiella* spp. (6.6%), *E. coli* (5.1%), *C. fruendii* (2.9%), *Pr. mirabilis* (0.7%), *Strep. pyogenes* (0.7%) and *H. influenzae* (0.7%). *Staph. aureus* isolated during the study were mostly susceptible towards cefoxitin (67.1%). Twenty three out of seventy *Staph. aureus* isolates (32.9%) were found to be MRSA. All the MRSA were susceptible to vancomycin. All the *Ps. aeruginosa* isolated were susceptible to imipenem and 92.3% of them were susceptible to piperacillin. Most of the isolated bacteria of enterobacteriaceae family were susceptible to gentamicin. The single *Strep. pyogenes* isolated was susceptible to erythromycin, amoxycillin, cefotaxime and chloramphenicol. The only *H. influenzae* isolate was susceptible to cefotaxime, ciprofloxacin and azithromycin but resistant to amoxycillin and chloramphenicol.

6.2 RECOMMENDATIONS

- Since, both Gram positive and Gram negative organisms could cause otits media, the antibiotics active against both group of bacteria should be used. *Staph. aureus* and *Ps. aeruginosa* were the most common bacterial pathogens observed in our study, aminoglycosides (gentamicin) or

fluoroquinolones could be prescribed as they are active against both the organisms.

- The commonly used antibiotic, amoxycillin was not active against most of the bacterial isolates obtained in our study. Similarly, another common antibiotic, ciprofloxacin was also found to be active against only minority of the isolates. So, the use of these antibiotics should be reviewed.
- The number of MRSA isolates was high in our study. Studies on epidemiological characterization of these isolates can be done to distinguish whether those strains are hospital acquired or community acquired.

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APPENDIX A

QUESTIONNAIRE AND PERFOMA

A. CLINICAL PROFILE:

Date:

Sample No:

Name:

Sex: Age:

Number of Siblings (for children):

Personal Hygienic Practice:

Short Clinical History:

History of Antibiotic Therapy:

Macroscopic examination of sample:

B. MICROBIOLOGICAL PROFILE:

Day 1

a. Direct microscopic examination

Gram staining: **Result**

a. Gram positive cocci
b. Gram positive bacilli
c. Gram negative bacilli
d. Gram negative cocci

b. Inoculation into different Agar Media

Day 2

a. Observation of Colony Characteristics on Different Media

Media Used:	**Type of Colony**
Nutrient agar (NA)	
MacConkey agar (MA)	
Blood agar (BA)	
Chocolate agar (CA)	

b. Gram Staining

c. Catalase Test

d. Oxidase Test

e. Inoculation to Biochemical Media

Day 3

a. Observation of Results in Biochemical media

Biochemical Test Employed	**Result**
a. Coagulase test	
i. Slide coagulase	
ii. Tube coagulase	
b. Triple Sugar Iron (TSI)	
c. Sulphide Indole Motility (SIM)	
d. Citrate Utilization	
e. Urea hydrolysis	

ORGANISM ISOLATED:

b. Antibiotic Susceptibility Test

Day 4

Antibiotic Susceptibility Profile

Antibiotics used	Zone of Inhibition	Remarks
a. Amikacin		
b. Amoxycillin		
c. Azithromycin		
d. Cefixime		
e. Cefotaxime		
f. Cefoxitin		
g. Ceftazidime		
h. Chloramphenicol		
i. Ciprofloxacin		
j. Co-trimoxazole		
k. Erythromycin		
l. Gentamicin		
m. Imipenem		
n. Piperacillin		

APPENDIX B

LIST OF MATERIALS

1. Equipments

Autoclave	Hot air oven
Candle extinction glass jar	Weighing balance
Burner	Microscope
Incubator	Refrigerator

Glass wares: Petri plates, tubes, slides, glass rod etc.

2. Microbiological media (Hi-Media)

Nutrient Agar	Simmon's Citrate Agar
Nutrient Broth	TSI Agar
Mac Conkey Agar	Blood Agar
Muller Hinton Agar	SIM Media
Urease Agar	

3. Chemicals/Reagents

Catalase reagent (3% H_2O_2)

Oxidase regent (1% Tetramethyl p-phenylene diamine dihydrochloride)

Crystal violet	Gram's iodine
Acetone-alcohol	Safranin
Blood plasma	Kovac's reagent

4. Antibiotic discs (Hi-media)

Amikacin (30 mcg)	Amoxycillin (10 mcg)
Azithromycin (15 mcg)	Bacitracin (10 mcg)
Cefixime (5 mcg)	Cefotaxime (30 mcg)
Cefoxitin (30 mcg)	Chloramphenicol (30 mcg)
Ciprofloxacin (5 mcg)	Co-trimoxazole (25 mcg)
Erythromycin (15 mcg)	Gentamicin (10 mcg)
Imipenem (10 mcg)	Piperacillin (100 mcg)

5. Miscellaneous

Inoculating loops, Straight wires, Cotton swabs, Distilled water, Immersion oil, Lysol, Oil, Dropper, etc.

APPENDIX C

COMPOSITION AND PREPARATION OF DIFFERENT TYPES OF MEDIA

I. Composition and Preparation of Culture Media

The culture media from Hi-Media Laboratories Pvt. Limited, Bombay, India were used.

(All compositions are given in Grams per liter and at 25^0C temperature)

1. Blood agar (BA)

Blood agar base (infusion agar) + 5-10% human blood

Ingredients	gm/liter
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0

Final pH (at 25˚C) 7.3±0.2

40 Grams of the blood agar base medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121˚C (15lbs pressure) for 15 minutes. After cooling to 40-50˚C, 50 ml sterile human blood was added aseptically and mixed well before pouring.

2. Chocolate agar (CA)

The blood agar plates prepared by the above mentioned process was placed in the hot air oven at the temperature 70˚C for 10-15 mins till the agar changed into chocolate brown colour.

3. MacConkey Agar (MA)

(Without crystal violet, sodium chloride and with 0.5% sodium taurocholate)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0
Neutral Red	0.04
Agar	20.0

Final pH (at 25˚C) 7.4±0.2

55 Grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121˚C (15 lbs pressure) for 15 minutes.

4. Mueller Hinton Agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Acid Hyrolysate	17.5
Starch	1.5
Agar	17.0

Final pH (at 25˚C) 7.4±0.2

38 Grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve. 10 ml was distributed in test tubes and sterilized by boiling in water bath for 10 minutes.

5. Nutrient Agar (NA)

Ingredients	gm/litre
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0

Final pH (at 25˚C) 7.4±0.2

37 Grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121˚C (15 lbs pressure) for 15 minutes.

6. Nutrient Broth (NB)

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5

Final pH (at 25˚C) 7.4±0.2

13 Grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121˚C for 15 minutes.

II. Composition and Preparation of Biochemical Test Media

1. Triple Sugar Iron (TSI) Agar

Ingredients	gm/litre
Peptic digest of animal extract	10.0
Casein enzymic hydrolysate	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0

Final pH (at 25˚C) 7.4±0.2

65 Grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121˚C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

2. Sulphide Indole Motility (SIM) medium

Ingredients	gm/litre
Beef Extract	3.0
Peptic digest of animal tissue	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0

Final pH (at 25˚C) 7.3±0.2

36.23 Grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121˚C for 15 minutes at 15 lbs pressure.

3. Simmon Citrate Agar

Ingredients	gm/litre
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Bromothymol Blue	0.08
Agar	15.0

Final pH (at 25˚C) 6.8±0.2

24.28 Grams of the medium was dissolved in 1000ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121˚C for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

4. Christensen Urea Agar

Ingredients	gm/litre
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0

Final pH (at 25˚C) 7.4±0.2

24 Grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at 121˚C for 15 minutes. After cooling to about 45˚C, 50 ml of 40% urea was added aseptically and mixed well. Then 5 ml was dispensed in test tube and set at slant position.

III. Staining and Test Reagents

1. For Gram's Stain

(a) Crystal Voilet solution

Crystal Voilet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml

Distilled Water (D/W) to make 1 litre

Preparation: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally the volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Preparation: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Preparation: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then, immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Preparation: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

2. Test Reagents

a. For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

b. For Oxidase Test

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

c. For Indole Test

Kovac's Indole Reagent	
Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Hydrochloric acid	10 ml

Preparation: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

APPENDIX D

GRAM-STAINING PROCEDURE

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal violet dye to be washed out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion.

APPENDIX E

BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

a. Catalase test:

During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. Catalase enzyme breaks down hydrogen peroxide into water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* sp.

A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g., Blood Agar) or if an iron wire loop is used.

b. Oxidase test:

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test.

A piece of filter paper was soaked with few drops of oxidase reagent. Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

c. Citrate Utilization test:

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. Organisms capable of utilizing citrate as its sole carbon source also utilizes the

ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media from green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

d. Motility test:

The motility media used for motility test are semisolid, making motility interpretations macroscopic. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

e. Triple Sugar Iron (TSI) Agar:

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium).

The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

f. Urea Hydrolysis test:

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia, thus produced, changes the color of indicator incorporated in the medium.

The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. Positive organism showed pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in colour of the indicator to pink.

g. Coagulase test:

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S aureus* (usually positive) from *S epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *Staphylococcus aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

Slide Coagulase Test:

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The

third drop of saline was used for a known strain of coagulase positive staphylococci.

Tube Coagulase Test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

In the tube coagulase test, plasma was diluted 1 in 10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*Staphylococcus aureus* culture), and 0.5 ml negative control (*Staphylococcus epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37^0C on a waterbath for 6 hours and observed for gel formation in every 30 minutes.

APPENDIX F

ZONE SIZE INTERPRETATION CHART

Antibiotic	Symbol	Strength	Diameter of zone of inhibition in mm		
			Resistant (mm or less)	**Intermediate (mm)**	**Sensitive (mm or more)**
Amoxycillin	AMX	10mcg			
For enterobacteriaceae			13	14-16	17
For Staphylococci			28	-	29
For *H. influenzae*			18	19-21	22
For *S. pyogenes*			18	19-25	26
Cefoxitin For Staphylococci	CX	30 mcg	21	-	22
Bacitracin	B	10 units	8	9-12	13
Chloramphenicol	C	30 mcg	25	26-28	29
For *H. influenzae*			17	18-20	21
For *S. pyogenes*					
Ciprofloxacin	Cip	5 mcg	15	16-20	21
For *H. influenzae*			-	-	21
Co-Trimoxazole	Co	25 mcg	10	11-15	16
Cloxacillin	Cx	5 mcg	11	12-13	14
Erythromycin	E	15 mcg	13	14-22	23
For Staphylococci			15	16-20	21
For Streptococci					
Gentamicin	G	10 mcg	12	13-14	15
Ceftazidime	CAZ	30 mcg	14	15-17	18
Amikacin	AK	30 mcg	14	15-16	17

Table continued					
Piperacillin For *P. aeruginosa*	P	100 mcg	17	-	18
Imipenem	I	10 mcg	13	14-15	16
Cefixime For enterobacteriaceae	CFX	5 mcg	15	16-18	19
Cefotaxime For *H. influenzae* For *S. pyogenes*	CTX	30 mcg	- 25	- 26-27	26 28
Azithromycin For *H. influenzae*	AZM	15 mcg	-	-	12

Manufacturers: HiMedia Laboratories Pvt. Limited, Mumbai, India.