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Effect of Tris-EDTA and ascorbate in increasing antibiotic activity against bacteria isolated from Otitis Media

A Thesis

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By

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بسم الله الرحمن الرحيم

وَعَلَّمَكَ مَالَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيماً

صدق الله العظيم

سورة النساء ١١٣

Dedication

To them who had made me of what I am…. My family and my husband, the cause of my success….

Arwa

Supervisors Certification

We certify that this thesis was achieved under our supervision in the College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

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Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as thesis for the degree of Master of Science in Biotechnology.

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Summary

The present study was carried out to shed light upon the some materials that used in increasing antibiotic activity against the isolated bacteria from otitis media patients.

A total of (84) samples were collected from different age group male and female patients with otitis media. The patients were either hospitalized at Al-Kadhumia Educational Hospital or attending the Central Public Health Laboratory of Ministry of Health during the period from 5/12/2006 - 28/5/ 2007.

A total 59 samples gave positive results, of which 25 were identified as *Pseudomonas areuginosa*, 15 *Staphylococcus epidermidis,* 6 *Staphylococcus aureus*, 5 *Escherichia coli***,** 3 *Proteus maribilis* and 5 fungi. *Pseudomonas areuginosa* was the most common pathogen in the studied samples of otitis media. Patients in the age range between (1-10) years were more infected than other age groups. Male patients were more infected than female patients with otitis media, and right ear was subjected to infection more than left ear. During winter, the frequency of infection was more than other seasons. The obtained results showed that single infection among patients with otitis media was more common mixed infection.

When susceptibility of bacterial isolates to 12 antibiotics was tested using disk diffusion assay. The results revealed that Ciprofloxacin was the most effective antibiotic against bacterial isolates followed by amikacin and then by gentamycin.

Nine isolates were selected according to their pattern of resistance as those showing muli-drug resistance and tested to specify their minimum

inhibitory concentration for (cephalexin, ciprofloxacin, streptomycin, amikacin, cefotaxim, tetracyclin, erythromycin, ampicillin, gentamycin, nalidixic acid). Ciprofloxacin was found to be the most effective one through its lowest MIC as compared to other antibiotics.

The effect of Tris-EDTA and ascorbic acid when were combined separately with antibiotics and tested against the nine isolates by using disk diffusion assay. The obtained results showed that there was a synergistic effect between Tris-EDTA or (ascorbic acid) and each of β- lactam, tetracycline, aminoglycosides**,** SXT, quinolone and erythromycin against Gram negative and positive bacteria. Adversely, there was an antagonistic effect between Tris-EDTA or ascorbic acid and quinolone or aminoglycosides against gram negative bacteria, only.

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List of Abbreviations

Abbreviation	Key
AOM	Acute Otitis Media
Api20E	Analytical Profile Index 20E
CSOM	Chronic Suppurative Otitis Media
DNA	Deoxyribonucleic acid
DNase	Deoxyribonucleic
IL1	Interlukin1
IL6	Interlukin6
Igs	Immunoglobulin
EDTA	Ethylenediamineteraactic acid
ESBLs	Extended spectrum β-lactamase
HCl	Hydrochloric acid
H_2O_2	Hydrogen Peroxide
MIC	Minimum Inhibitory Concentration
mM	MilliMolar
O.D.	Optical density

OM	Otitis Media
PBPs	Penicillin–Binding–Proteins
rRNA	Ribosomal nucleic acid
tRNA	Transfer ribonucleic acid
TNF	Tumor Necrosis Factor
TSI	Triple Sugar Iron
URTI	Upper Respiratory Tract Infection
UTI	Urinary Tract Infection
U.V	Ultra violet light
γ-INF	Gamma Interferon

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Chapter One

Introduction And Literatures Review

Introduction and Literature Review

1.1 Introduction

Otitis media is known to be one of the most common childhoods infections and a leading reason for antibiotic prescriptions in the development world (Bingen, 1998).

There are two types of otitis media: Acute otitis media which is defined as the presence of middle ear effusion in conjunction with the rapid onset of one or more signs or symptoms of inflammation of the middle ear (Friedman, *et al*., 2006), while other type which is known as Chronic suppurative otitis media is characterized by persistent perforation of tympanic membrane with recurrent or persistent otorrhea (Ologe and Nwawolo, 2002).

The pathogenesis of otitis media is multi-factorial, including infection, impaired Eustachian tube function, immature immune statue and allergy (Darrow, *et al*., 2003).

Antimicrobial drugs may either kill microorganism or inhibit their growth. Those that inhibit growth are called bacteriostatic, those drugs depend on the normal host defense to kill or eliminate the pathogen after its growth has been inhibited. Drugs that kill bacteria are bactericidal effect, these drugs are particularly useful in situation in which the normal host defenses can not be relied on to remove or destroy pathogen.

Consequently minimum inhibitory concentration are specified as that bacteriostatics effect that prevent visible growth of microbes on culture medium, application of such concentration were also important for resistance development and spread are a function of dosage of applied antibiotics.

Combinations of antibiotics and other substances (acts as drugs) are sometimes used to treat infections, but care must be taken when selecting the combination because some drugs will counteract the effect of another. When

the action of one drug enhances the activity of another, the combination is called synergistic. In contrast, combination in which the activity of one interferes with the other is called antagonistic. Combination that are neither synergistic nor antagonistic are called additive.

Aims of Study

- Isolation and identification of bacteria causing otitis media.
- Determining the antibiotics susceptibility patterns of the isolates, in order to select the appropriate antibiotics for MIC determination.
- Investing the effect of Tris-EDTA and ascorbic acid in increasing the inhibition activity of antibiotics against the isolated bacteria.

1.2 Literatures Review

1.2.1 The Ear

The organ of hearing and balance are divided into three parts: external, middle and inner ear. The external ear is the part extending from the outside of the head to the eardrum, the middle ear is an air-filled chambers medial to the eardrum, the inner ear is a set of fluid-filled chambers medial to the middle ear (Seeley *et al*., 1996) (Figure 1-1).

The middle ear is part of a system that includes the nares, the nasopharynx, the Eustachian tube and the mastoid air cell, and these structures were lined with respiratory epithelium. The middle ear communicates with the nasopharynx through the Eustachian tube interiorly and the mastoid antrum posteriorly (Hall and Colman, 1975).

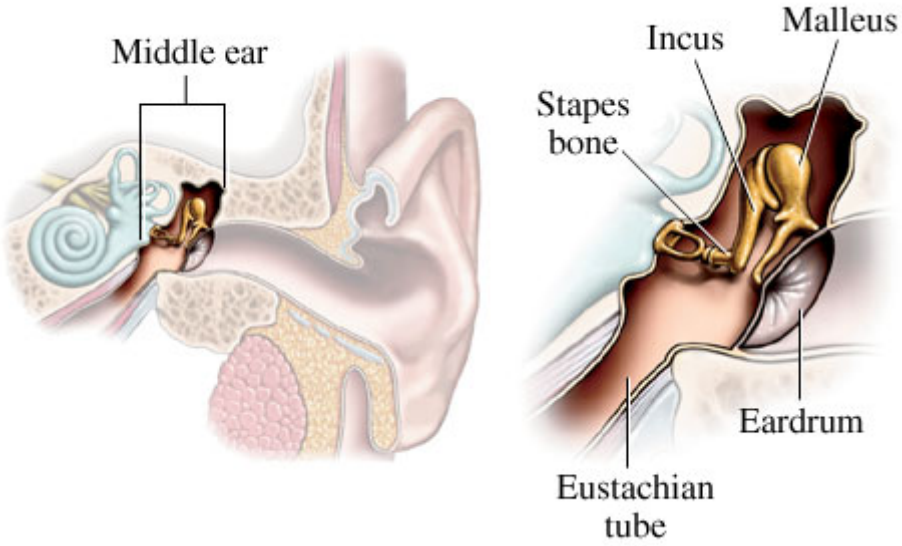


Figure (1-1) Structure of ear.

1.2.2 Otitis Media (Middle Ear Infection)

Otitis media is a general term used to describe any inflammation process involving the middle ear cleft (Jung and Hanson, 1999). Three types were recognized: Acute Otitis Media (AOM), Silent Otitis Media and Chronic Suppurative Otitis Media (CSOM) (Paparella *et al*., 2001). Turgut, *et al*. (2004) considered the Silent otitis media as transitional stage between Secrotery Otitis Media and Chronic Suppurative Otitis Media and its occur with complications of otitis media such as meningitis.

Otitis media can affect anyone, but mostly affect children (Jones, 2004). Children particularly prone to Otitis Media because their Eustachian tube is shorter, more horizontal and more compliant than those of older person (Roddey and Hoover, 2000).

Otitis media usually appear after Upper Respiratory Tract Infection (URTI), that disrupts the function of Eustachian tube, (the Eustachian tube dysfunctions which may begin a sequence of events that may lead to chronic ear disease), it is found that 40% of children susceptible to this infection through their first five years of their age (Jones, 2004), it leads annually to the death of over 50 000 children under five years (Rovers, 2006).

Eustachian tube has three major functions (Ronald, 1999):

a. Protection from nasopharyngeal sound pressure and secretion.
b. Clearance into the nasopharynx of secretions produced within the middle ear.
c. Ventilation of the middle ear to equilibrate air pressure in the middle ear with atmospheric pressure and to replenish oxygen that has been absorbed.

Impairment of each of these functions may result in acute otitis media (Adderson, 1998).

1.2.3 Mechanism of Otitis Media Infection

The infection of Otitis Media induced by endotoxin or other bacterial products, particularly cell wall products, and also by local allergens, this leads to the release of cytokines including Tumor Necrosis Factor (TNF), Interlukin1 (IL1) and Interlukin6 (IL6) which trigger the inflammatory response which in turn attract and activate white blood cells (Kenna, 1998).

The inflammatory response creates mucosal edama, capillary engorgement and polymorphonuclear leukocytes infiltration into the lamina propria of the mucosa of the pneumatized spaces of the middle ear. Purulent exudates soon fills the spaces, epithelial ulceration may occur, allowing granulation tissue proliferation which may help to maintain the infection, obstruct drainage and ventilation, and enzymatically destroy bone, the granulation tissue which can develop into polyp within the middle ear space (Roland *et al*., 2005).

1.2.4 Acute Otitis Media (AOM)

Acute Otitis Media is a disease of infancy and childhood occurs between the eardrum and the inner ear (Koivunen *et al*., 2004). AOM is inflammation of fluid in the middle ear (Takata *et al*., 2001), accompanied by the rapid onset of symptoms and signs of an ear infection including: inflammation or bulging of the tympanic membrane, otalgia (Pichichero, 2000), deafness in the affected ear, fever, blood, pus coming out of the ear if the ear drum ruptures (Simon *et al*., 2002), earache, anorexia, nausea and vomiting (Mandle *et al*., 2002).

Acute Otitis Media may be a result of infection with viral, bacterial or both. Twenty to thirty percent of episodes of AOM are caused by viruses (McCormick *et al*., 2000).

In 1989, Teel and his colleagues suggested that OM is over diagnosed in North America, as it is said that 84% of children have at least one episode of AOM by the first three years of life, while, Ross (1988) found that the incidence is approximately 70% in United Kingdom.

Another study indicated that 60% of children who had their first episode of AOM before the age of six months have two or more recurrence in 2 years. The earlier the age of onset of AOM, the greater the recurrence rate. (Pelton, 1996).

Persistent effusion is seen after AOM in 50% of children one month post AOM, 20% at two months, 10% at three months. The earlier the onset of AOM, the greater likelihood of persistent effusion. Persistent fluid in the middle ear is associated with conductive hearing loss, and can hinder language development and school performance (Pelton, 1996).

The most frequent causative agent of AOM is *Streptococcus pneumonia* 40%, followed by non typeable *Hemophilis influenzae* 25%, *Moraxella catarrhalis* 10%, Group A *Streptococcus* 2% and *Staphylococcus aureus* 2% (Stephenson *et al*., 1992).

1.2.4.1 Microbiology of Acute Otitis Media

The predominant pathogens of AOM were *S. pneumonia*, *H. influenzae* and *M. catarrhalis* (Rovers *et al*., 2004), and causes much type of infections such as: sinusitis, bacteremia, pneumonia, as well as meningitis (Kaijalainen *et al*., 2004).

Streptococcus pneumonia is a gram positive diplococcal. It is the most important cause of AOM and accounts for about 30% to 40% of cases (Matar *et al*., 2001). It is among the most important bacterial pathogens in infants and children, and infection caused by *S. pneumonia* is a major cause of morbidity and mortality (Jakobsen *et al*., 2001).

Hemophilis influenzae produces a wide range of diseases in many organ systems. It is small, gram negative, none sporulating, pleomorphic rod or coccobacilli (Wintrobs *et al*., 1974). It's the second most frequent isolated bacterium and is responsible for approximately 20% of episodes in preschool children (Donaldson, 2006).

Moraxella catarrhalis is the major causes of Acute and Chronic otitis media (Fadon *et al*., 1997), and also can causes other types of infections such as sinusitis and Respiratory Tract infection in both children and adult (Hays *et al*., 2003). It is responsible for aboute 10% of cases (Donaldson, 2006).

Other bacteria isolated from children with AOM were Group A *Streptococcus* with range 0-11%, is gram positive coccus, primarily is a pathogen of the pharynex (Maxon and Yamauch, 1996).

Viruses seem to interact with bacteria and enhance the local inflammatory process in middle ear (Heikkinen and Chonmaitree, 2003).

Viruses, alone or in combination with bacteria pathogens can be isolated from about 20% of AOM cases (Adderson, 1998).

Heikkinen and his collogues (1999) have found that Respiratory syncytial virus is the most commonly identified virus, being found in 47% of the middle ear isolates, and followed by parainfluenza virus and influenza virus.

1.2.5 Chronic Suppurative Otitis Media

Chronic Suppurative Otitis Media (CSOM) is defined as a chronic inflammation of the middle ear and mastoid mucosa in which the tympanic membrane is not intact and discharge is present (Bluestone, 1996).

Chronic Suppurative Otitis Media may be caused by the failure of an acute infection to resolve completely or by the infection of cholestoma or of serious effusion in the middle ear and is absence of acute inflammation

(Brook *et al*., 1992). It is characterized by recurrent or persistent ear discharge (otorrhoea) over two weeks' duration in the presence of tympanic membrane perforation (Active), or with dry but permanent perforation of the tympanic membrane (Inactive) (Acuin, 2004).

Chronic Suppurative Otitis Media causes loss of hearing for 50% of patients (Choa and Wu, 1994). Some studies showed a relation between the losses of hearing during the first year's childhood with infection of the middle ear, this lead to retardation of the child education (Fliss *et al*., 1990). The episodes of acute infection lead to cause a CSOM. The distribution of CSOM is less than AOM (Kenna, 1988).

The factors that led to an increase in high levels of CSOM are the continuous or recurrent infections of the middle ear with AOM, also usage of unsuitable antibiotics, continuous Upper Respiratory Tract infections URTI, nose disease and malnutrition (Jahn , 1991).

1.2.5.1 Microbiology of Chronic Suppurative Otitis Media

Several bacterial isolates were found associated with CSOM, however, Vartiainen and Vartiainen (1996) found that the most common bacterial species where *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Qguntibeju (2003) showed that the most commonly identified pathogenic bacterial species was *P. aeruginosa*. While, Melaku and Lulseged (1999) showed in their research that *Proteus* the more common bacteria 40(37%) from 80 patients and other researchers showed that the more common bacteria that causes ear discharge was *Staphylococcus aureus* 34.3% (Jha *et al*., 2007).

1.2.5.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is Gram negative, bacilli, measuring about (1.5 – 3.0X0.5µm) in length, usually motile by virtue of one or more polar flagella, non sporing and non capsulate (Cruickshauk *et al*., 1975). It is strict aerobe, occurs as single bacteria, in pairs and occasionally in short chains, belonging to the bacterial family *Pseudomonadaceae* (Jawetz *et al*., 1998).

Ps. aeruginosa is able to secrete several toxic proteins that are thought to act as virulence factors and these proteins after colonization can cause extension tissue damage and blood stream invasion (Todar *et al*., 2004).

Exotoxin A is a potentially important virulence factor of *Ps. aeruginosa*, which has the same mechanism of diphtheria toxin. Exotoxin A is responsible for local tissue damage, bacterial invasion and immunosuppression (Wood *et al*., 1989).

Another potentially extracellular enzyme produced by most isolates of *Ps. aeruginosa* is Exoenzyme S, which is responsible for tissue distribution and impairing the function of phagocytes in blood stream and organs to prepare for invasion by *Ps. aeruginosa* (Brint *et al*., 1995).

Ps. aeruginosa produce three other soluble proteins involved in invasion, cytotoxin and two haemolysins (Van-Delden and Igleweski, 1998). The cytotoxin is a pore forming protein, it was originally named leukocidin because of its effect on neutrophilis but it showed cytotoxic for eukaryotic cells, the two haemolysins, first is phospholipase and the other is lecithenase, they appear to act together to break down lipid and lecithin (Todar, 2004).

Ps. aeruginosa also produce two exoproduct, protease (cause lyses of fibrin) and elastase (causes destruction of immunoglobulin (Igs) and other compliments, and also lyses fibronectin) during infection, in order to facilitate the invasion and dissemination of these bacteria. The two enzymes act

together to destroy the structure of fibrin and elastin and cause inactivation of gamma interferon (γ-INF) and TNF (Todar, 2004).

Also there is a lipopolysaccharide that is responsible for many endotoxic properties and exopolysaccharide (alginate) which is antiphagocytic (Brook *et al*., 1995).

1.2.5.1.2 *Proteus mirabilis*

This bacterium belongs to family *Enterobactericeae*, a gram negative, rod measuring 1.5-3.0μm in length and 0.5μm in diameter, actively motile and non capsulated (Levinson and Jawtez, 2000). It is produce urease which considered as virulence factor resulting in rapid hydrolysis of urea with liberation of ammonia, lipase production, hemolysin production, protease production and also contain lipopolysacchrids is considered as enterotoxin (Swierzko *et al*., 2000).

The genus *Proteus* has four species which are *Pr. mirabilis*, *Pr. vulgaris*, *Pr. pneneria* and *Pr. myxofaciens* (Jawtes *et al*., 1998). The most important feature which differentiat *Proteus* from other genera of the *Enterobactericeae* is swarming phenomena (Iwalokun and Akinwvmi, 2002).

Pr. mirabilis can be distinguished from other three species by its inability to form indole (Wintrobe *et al*., 1974). It is considered as opportunistic bacteria produced infections in human only when the bacteria leave the normal habitat (intestinal tract) and produce otitis media, bacterimia, pneumonia and Urinary Tract Infection UTI (Zhanel *et al*., 2000). The great danger of these infections lies in intracranial extension, leading to thrombosis of the lateral sinus, meningitis, brain abscess and bacterimia (Wintrobe *et al*., 1974).

Pr.mirabilis produces many toxic proteins: a) two distinct hemolysins which are Hpm A and Hly A (Senior, 1997), b) protease that cleaves IgA1

and IgA2 (Senior, 1999), c) proteocine (Tracy and Thomson, 1972), d) outer membrane protein and has lipopolysacchried (Swierzko *et al*., 2000)

1.2.5.1.3 *Staphylococcus aureus*

Staphylococcus aureus is a gram positive bacteria, of about one micrometer diameter. The cocci are mainly arranged in grape like clusters. The organisms are non spore forming, non motile and usually non capsulated. When grown on solid media for 24 hours at 37°C, individual form circular colonies, two to three millimeter in diameter with a smooth shiny surface. Colony appearance is often pigmented (golden, yellow, buff or cream), *S. aureus* are salt tolerant (Cruickshank *et al*., 1975).

Enzymes produced by *Staphylococcus* include catalase, coagulase, hyaluronidase, proteinase, lipase and β-lactamase. (Somervill *et al*., 2002).
Also, *Staphylococcus aureus* have the ability to produce enterotoxin and exotoxin.

There are other microorganisms that cause CSOM mentioned as followes:

Escherichia coli, Klebsiella spp. and Serratia spp. are gram negative rod that belongs to *Enterobacteriaceae*. The most members of *Enterobacteriaceae* considered as the normal habitat in intestinal tract and causes infections when the bacteria leaves it, such as otitis media, Respiratory Tract infection, pneumonia and meningitis (Tanabe *et al*., 2005).

Staphylococcus epidermidis was belonging to family *Micrococcaceae,* it is coagulase negative and mannitol no fermenting (Wang *et al*., 2003). It is considered as member of normal flora of human skin, respiratory and gastrointestinal tracts (Lowy, 1998), but became opportunistic when there is a decreases in body resistance and causes many infections such as otitis media (Stuart *et al*., 2003).

Acinetobacter baumannii is gram negative, facultative aerobic that grow at 20-37°C (Holt *et al*., 1994), this bacteria is opportunistic that affect different body tissues (Garcia *et al.*, 1999), also resistant for high range of antibiotics (Martro *et al*., 2003).

Candida spp. and *Aspergillus* spp., the most causatives of fungal infection, especially for adults aged between (16-30) year (Yehia *et al*., 1990). The signs and symptoms of fungi are: discharge, loss of hearing, acute headache and redness of skin (Kurnatowski and Filipiak, 2001).

1.2.6 Complications of Otitis Media

1.2.6.1 Acute Intratemporal Complications

The Acute Intratemporal Complications of otitis media include mastoiditis (acute Suppurative infection of mastoid gas cell system), labyrinthitis, facial paresis and external otitis (Goldstein *et al*., 1998).

1.2.6.2 Acute Intracranial Complications

The Acute Intracranial Complications of otitis media include meningitis, focal encephalitis, extradural abscess, subdural empyema, brain abscess, sinus thrombosis and otitic hydrocephalus (Bluestone and Klein, 2001).

1.2.7 Risk Features of Otitis Media

1.2.7.1 Host Risk Features of Otitis Media

Host risk features provide prognostic information and identify children who may benefit from preventive program (Klein and Bluestone, 1997).

a. Age

Ear infection occurs in all age groups, but it's considerably more common in children particularly those aged six months to three years than in adults. This age distribution is presumably due to immunologic factors (e.g. lack of pneumococcal antibodies) and anatomic factors (e.g. a low of Eustachian tube with relation to the nasopharynx) (Lubianca *et al*., 2006).

b. Birth weight and gestational age

A recent study from the Netherlands suggests that gestational age of less than 33 weeks and very low birth weight (less than 1500 gm) are risk factors for recurrent OM (Klein and Bluestone, 1997).

c. Sex

Varying distribution among sex has not found, some studies have shown an increased preponderance in males when compared to female (Marchant *et al*., 1992; Kenna, 2000; Lubiancn *et al*., 2006), but others studies revealed an equal distribution (Kenna, 2000).

Boys had a 1.5 times greater risk for recurrent disease than girls (Klein *et al*., 1992).

d. Race

The Americans Indian and Eskimo demonstrate an increased risk of infection. The anatomy and function of the Eustachian tube play a significant role in this increased risk, the Eustachian tube wider and more open in these populations than in others (Parry, 2006).

e. Allergies

Allergies may contribute to ear infections, possibly by increasing the amount of fluid in the middle ear. There is some evidence that children allergic to pollens, dust, molds and foods may be more likely to develop AOM (Hurst, 1996).

1.2.7.2 Environmental Features of Otitis Media

Many Environmental Features have been identified as important in the development of OM.

a. Day care attendance

Day care attendance has been associated with an increased incidence of AOM (Teele *et al.*, 1989; Pelton, 1996). Some studies reported that care outside the home increases the relative risk of recurrent AOM (Adderson, 1998), it is two times more likely a child in day care centers will have frequent ear infection compared with a child cared for at home (Marx *et al*., 1995).

b. Exposure to tobacco smoke (passive smoking)

Environmental tobacco smoke may be an important risk factor for middle ear disease (Adair-Bischoff and Sauve, 1998). A study of day care students found that the children exposed to cigarette smoke at home had higher risk of middle ear infections than the children whose parents didn't smoke (Etzel *et al*., 1992).

c. Breast feeding

Breast feeding has been suggested as an important factor in prevention of Respiratory tract infections and middle ear disease in infancy. Many study

shown an inverse relationship between the incidence of middle ear disease and the duration of breast feeding (Bluestone and Klein, 1983). The study that showed breast feeding for a period as short as even 3 months decreased the incidence of AOM in the first year of life (Klein, 1994).

d. Season

The incidence of OM parallels the incidence of upper Respiratory Tract infections (Klein and Bluestone, 1997), which are most prevalent during the winter months (Hoberman and Paradic, 2000). OM also common in the spring and fall, and least common during the summer (Deskin, 1999).

1.2.8 Antibiotic Treatment of Otitis Media

The choice of antibiotics should be taken into consideration their *in vitro* activity against prevalent organisms. Appropriate choice of antimicrobial agent for therapy of OM is based on the understanding of the microbiology of the acute and chronic disease.

1.2.8.1 β-lactam Antibiotics

β-lactam antibiotics irreversibly inhibit enzymes involved in the final steps of cell wall synthesis, the enzymes inhibited by β-lactam drugs mediate the formation of peptide bridges between adjacent strands of peptidoglycan which called penicillin–binding–proteins (PBPs) (Nester *et al*., 2001). All members of this group have a shared chemical structure called β-lactam ring (Chambers *et al*., 2001).

β-lactam include two groups: Penicillin and Cephalosporines. The Penicillin discovered by Alexander Fleming in 1928, isolated from *Penicillum*

notatum in 1929 (Atlas, 1995). Penicillin group is the most widely used group of antibiotics due not only to their action but for their stability to gastric acid, thus, they are suitable for oral administration, and also for their possessing less toxicity than the most of the other antibiotics (Jawetz *et al*., 1998).

Ampicillin and amoxicillin are given orally to treat otitis, UTI, and lower respiratory infection, they are active against Gram negative such as: *E.coli* and *P.mirabilis,* but they are inactivated by β-lactamases enzyme (Cercerado *et al*., 1990), They have the same spectrum and activity but amoxicillin is better absorbed from the gut, less making diarrhea and also give high blood level (chambers *et al*., 2001).

A combination of amoxicillin with β-lactamase inhibited clavulanic acid, that interfere with the activity of some types of β-lactamases and thus amoxicillin is protected against enzymatic destruction (Chambers *et al*., 2001 and Nester *et al*., 2001).

Cephalosporines are derived from an antibiotic produced by the fungus called *Cephalosporium acremonium* (Peltier *et al*., 2004); Cephalosporines were originally introduced to use in patients with allergy to penicillins (Davis *et al*., 1990).

Cephalosporines are divided into four major generations depending mainly on the spectrum of antimicrobial activity (Chambers *et al*., 2001).

First generation cephalosporines include: cephalexin, cephalothin, cefadroxil, cefazolin, and cephradine, are highly active against Gram positive bacteria and are moderately active against some Gram negative rods such as *E.coli*, *Proteus*, and *Klebseilla* (Mitsuhashi, 1980).

Second generation cephalosporines include: cefaclor, cefuroxime, cefprozil, loracarbef, and cefonicid. All these antibiotics are less active against Gram positive bacteria than first generation (Chambers *et al*., 2001),

but are more active against Gram negative rods, such as *E.coli* , and *Proteus* (Jawetz *et al*., 1998).

The third generation of cephalosporines has major advantages as their expanded Gram negative coverage and the ability of some to cross the blood brain barrier (Chambers *et al*., 2001).

Cefepime is an example of fourth generation cephalosporines, it is more resistant to hydrolysis by chromosomal β-lactamases, and some extended–spectrum β-lactamases that inactivate many of the third generation cephalosporines (Chambers *et al*., 2001).

1.2.8.1.1 Resistance to β-lactam

The resistance of bacteria to β-lactam is due to degradation of drug by β-lactamase which is produced by bacteria, lack or poor permeability to the drug by bacterial cell and lack or altered PBPs which are drug receptors on the cell wall and some of these are enzymes involved in transpeptidation reaction (Nester *et al*., 2001).

The major basis for bacterial resistance to penicillines is the inactivation of drug by β-lactamase, which inhibit the activity of β-lactam antibiotics by breaking the β-lactam ring of antibiotic (Arakwa *et al*., 1989).
The inheritance of β-lactamase is either encoded by chromosome or plasmid (Wiedemann, 1990).

β-lactamase encoded by chromosome was observed in most Gram negative bacteria, while β-lactamase such as, penicillinase is encoded mostly by plasmid in *S.auerus* bacteria (95%of *S.aureus* producing plasmid encoded β-lactamase), however some strains produce both types of β-lactamase (i.e. chromosomal or plasmid mediated) (Hardy, 1986).

It was found that members of *Enterobacteriaceae* such as, *E.coli*, *Klebsiella,* and *Proteus mirabilis* have the ability to produce plasmid encoded

enzymes called Extended Spectrum β-lactamase (ESBLs) which enable the bacteria to resist most active β-lactams which are third generation cephalosporines, in addition at least six chromosomally mediated β-lactamases have been distinguished in ampicillin resistant *E.coli* by isoelectric focusing (Luzzaro *et al*., 2001).

Resistant to β-lactam spreads among member of *Enterobacteriaceae* by plasmids. Morever, Rassol, *et al*. (2003) successes in transfer the resistant to β-lactam from *Kebsiella pneumoniae* to *E.coli* by transconjugation.

1.2.8.2 Aminoglycosides

Aminoglycosides are group of bactericidal antibiotics (Dupont *et al*., 2000); originally obtained from various *Streptomyces* species, have a hexose ring, to which various amino sugars are attached by glycosidic linkages (Chambers *et al*., 2001).

All aminoglycosides inhibit protein synthesis by attaching to and inhibiting the function of 30S subunit of bacterial ribosome (Peltier *et al*., 2004). Streptomycin is considered as the oldest aminoglycoside drug which is still drug of choice for treatment of bacteria resistant to other types of drugs; however common Gram negative bacteria develop resistant to this drug (Mingeot-lecterco *et al*., 1999).

Amikacin is one of the important drugs which belong to aminoglycoside group, many Gram negative enteric bacteria including *E.coli*, *Proteus*, *Serratia*, and *Pseudomonas* are inhibited by amikacin, which are resistant to many enzymes that inactivate gentamicin and tobramicin (Chambers *et al*., 2001).

1.2.8.2.1 Resistance to Aminoglycosides

The resistance to aminoglycoside was due to enzymatic destruction (inactivation) of drug (plasmid mediated transmissible resistance), alteration

or deletion of ribosomal receptor (chromosomal mutation), and impaired entry of aminoglycosides into cell (Chambers *et al*., 2001).

In clinical isolates of Gram negative organism, resistance to aminoglycosides is due to the production of enzyme that specifically modify the antibiotic, so that it can no longer gain entry into the bacterial cell (Joklik *et al*., 1984). The enzymes inactivate the aminoglycosides by: acetylation of amino groups (acetyltransfrase), phosphorylation of hydroxyl group (phosphortransferase), or adenlylaion of hydroxyl group (adenyltransferase) (Forbes *et al*., 2002).

1.2.8.3 Quinolones

The mode of action of all quinolones is blocking bacterial DNA synthesis by inhibiting bacterial DNA gyrase, which prevents the relaxation of positively supercoiled DNA that is required for normal transcription and replication (Chambers *et al*., 2001).

Quinolones include many antibiotics such as: Naldixic acid, ciprofloxacin, norfloxacin, and ofloxacin (Pimental *et al*., 1998).

Quinolones are potent bactericidal agents; they have a broad spectrum of activity that includes Gram negative and gram positive bacteria (Forbes *et al*., 2002).

Ciprofloxacin and nalidixic acid are effective antibiotics in OM (Quinn, 2002). Ciprofloxacin has become one of the most widely prescribed antimicrobial agents and has low incidence of side effects (Mulhall and Bergann, 1995).

1.2.8.3.1 Resistance to Quinolones

Chromosomal resistance to Quinolones is developed by mutations which either cause an alteration in the A subunit of the target enzyme (DNA

gyrase) or change in outer membrane permeability, which on other hand result in decrease of drug accumulation (Forbes *et al*., 2002).

It was found that resistance to naldixic acid and ciprofloxacin, could be encoded by plasmid which could be transferred between *Enterobacteriaceae* and *P. aeroginosa* (Martinez – Martinez *et al*., 1998).

1.2.8.4 Macrolides

The macrolides are group of closely related compounds characterized by a macro-cyclic lactone ring; the erythromycin (representative of this group) was obtained in 1952 from *Streptomyces erythreus* (Chambers *et al*., 2001).

Macrolides have been widely used to treat various infections; they bind to 25S rRNA on the 50S ribosomal subunit resulting in blockage of transpeptidation and or translocation resulting in inhibition of protein synthesis (Kawamura-sato *et al*., 2000). The antimicrobial activity of macrolides is broad spectrum; thus macrolides antibiotics are used against Gram positive and some Gram negative bacteria (Jawetz *et al*., 1998).

Macrolides are especially useful for their excellent penetration of orophryngeal secrations and tissues (Quinn, 2002).

1.2.8.4.1 Resistance to Macrolides

Resistance to erythromycin result from an altered rRNA receptor, this is caused by chromosomal mutation, or under control of transmissible plasmids, commonly found in *S.auerus* and *Streptococcus* species (Chambers *et al*., 2001).

1.2.8.5 Tetracyclines

The tetracyclines are large group of drugs with a common basic structure and activity, tetracyclines obtained by catalytic dehalogenation of chlortetracycline, which (chlortetracycline) is isolated from *Strptomyces aureofaciens* (Chambers *et al*., 2001).

Tetracycline is concentrated intracellularly by sensitive bacteria and as a result protein synthesis is stopped by inhibits binding of aminoacyl-tRNA to the 30S subunit of bacterial ribosome (Joklik *et al*., 1984).

Tetracyclines are broad spectrum antibiotics and bacteriostatic antibiotics for many Gram positive bacteria and several intracellular bacterial pathogens such as *Chlamydia*, *Rikettsia* (Forbes *et al*., 2002).

1.2.8.5.1 Resistance to Tetracyclines

The most important mechanisms of resistance to tetracycline is by decreasing intracellular accumulation due to either impaired influx, or increased efflux, by an active transport protein pump, this pump protein encoded by transmissible plasmid (Chambers *et al*., 2001; Nester *et al*., 2001).

The mechanisms have been included in tetracycline resistance were: enzymatic inactivation of tetracycline, and ribosomal protection by interfering with tetracycline binding to the ribosome (Chambers *et al*., 2001). Chopra in 1985 found that the wide use of tetracycline result in spread bacterial resistance in most *Enterobacteriaceae*.

1.2.8.6 Trimethoprim-sulfamethaxazol (SXT)

Frequently trimethoprim is combined with a sulfonamide (usually sulfamethaxazol) to produce bactericidal agent that can simultaneously attack

two targets (competition with enzymes) on the same folic acid metabolic pathway (Forbes *et al*., 2002).

1.2.8.6.1 Resistance to Trimethoprim-sulfamethaxazol

Most common cause for resistance is due to production of resistant enzymes to both antibiotics, which are often under plasmid control (Chambers *et al*., 2001).

1.2.9 Substances Increase Antibacterial Activity

Some bacterial infection were extremely difficult to treat, this is attributed to high resistance of the organism to antibiotics. The primary cause of this high antibiotic resistance is reduced outer membrane permeability (Angus *et al*., 1982).

1.2.9.1 Tris-EDTA and its Antimicrobial Effect

Tris-EDTA acts as a chelating agent and enhances activity of antibiotics against pathogens by decreasing stability and increasing permeability of cell wall (Foil, 2004); it has been shown in vitro to have potent bactericidal effects (Gotthelf, 2005).

In vitro studies have shown that Tris-EDTA is less effective ininhibiting gram positive than gram negative bacteria, this may be because gram positive have less phospholipids and more peptidoglycan in cell wall compared with gram negative bacteria (Paterson, 2003).

EDTA a divalent cation chelator which removes Mg^{+2}, Ca^{+2} from outer membrane sites, lead to the release of cell wall lipopolysacchrides, protein

and other cell contents (Nicas and Hancock, 1983 and Foster and DeBore, 1998).

Tris is capable of potentate the action of EDTA in organism (Hancock, 1981), and acts as a buffer to keep the ear cannel at pH 8.0 which is optimum for function of aminoglycosides and fluroquinolones (Gotthelf, 2005).

1.2.9.2 Ascorbic Acid (Vitamin C)

It was first isolated in 1928, water-soluble, unstable compound, and can be destroyed by oxygen, alkali and high temperature (Iqbal *et al*., 2004).

Ascorbic acid has antibacterial, antiviral activity and is required to stimulate the immune function (activate phagocytic leukocytes) (Pauling, 1974), decrease the severity and duration of an infection (Hemila, 1992) and reduce inflammation that caused by bacterial infection (Brown, 1996).

Ehrismann (1942) found that strict aerobes were generally inhibited, suggesting that the inhibition was due to a reduction in the oxidation/ reduction potential of the medium. Some other researchers concluded that the inhibition was due to hydrogen peroxide formed during the auto-oxidation of ascorbic acid (Lwoff and Mo, 1942b).

Cathcart (1991) suggested that massive doses of ascorbic acid worked synergistically with appropriate antibiotics when used against acute bacterial diseases and considerably broadened the activity spectrum of the antibiotics.

Chapter Two

Materials And Methods

Materials and Methods

2.1 Materials

2.1.1 Apparatus and Equipment

The following Apparatus and Equipment used in the study:

Equipment	Company(Origin)
Autoclave	Gallenkamp (England)
Anaerobic jar	Rodwell (England)
Balance	Ohaus (France)
Distillator	Gallenkamp
Hot plate with magnetic stirrer	Gallenkamp
Incubator	Gallenkamp
Compound light microscope	Olympus (Japan)
Micropipette	Witeg (germany)
Millipore filter unit	Millipore and Whatman (England)
Oven	Memmert(germany)
pH-meter	Meter-GmPH Tdedo (England)

2.1.2 Culture Media

The following Culture Media used in the study:

Medium	Company (Origin)
Blood base agar	Mast-dignostic (England)
Chocolate agar	Prepared freshly in the laboratory
DNase agar	Difco U.S.A)
MacConky's agar	BioMark (India)
Mannitol salt agar	Oxiod (England)
Muller-Hinton agar	Biokit S.A (Spain)

Nutrient agar	BioMark
Nutrient broth	Oxoid (England)
Peptone water medium	Difco
Simon citrate media	Difco
Triple suger iron agar	Difco
Trypton	Difco
Urea agar base	Oxoid

2.1.3 Chemicals

The following Chemicals used in the study:

Chemical agent	Company (Origin)
Agar-agar	Fluka (Switzerland)
Crystal violet	BDH (England)
EDTA	Fluka
Glycerol	BDH
Hydrogen peroxide	BDH
Hydrochloric acid	BDH
Iodine	BDH
mannitol	Difco (U.S.A)
Phenol red	BDH
Tris	
Urea	BDH

2.1.4 Api20E kit (Api Bio merieux, lyon, France):

Api20E kit consists of:

a) The galleries:

The gallery is a plastic strip with 20 micro-tubes containing dehydrated reactive gradient.

b) Api20E Reagents:

i. Oxidase reagent (1% tetra-methyl-p-phenylene diamine).
ii. Kovac's reagent (p-dimethyl amino benzaldehyde at 4% in HCL isoamyle alcohol).
iii. Voges-Prskaver reagent:
   a) VPI (40% potassium hydroxid).
   b) VP2 (6% alpha-nephol).
iv. Ferric chloride (3-4%).

2.1.5 Antibiotic Disks (Bio analyze /Turkey)

The following Antibiotic Disks used in the study:

Antibiotic	**Code**	**Conc.(μg)**
Ampicillin	AM	10
Amoxicillin+clavulanic acid	AMC	20/10
Amikacin	AK	30
Cefotaxime	CXT	30
Cephalexin	CL	30
Ciprofloxacin	CIP	5
Erythromycin	E	15
Gentamicin	CN	10
Naldixic acid	NA	30
Streptomycin	S	10
Tetracycline	TE	30
Trimetheprim+sulph	SXT	1.25/23.75

amethoxazole		

2.1.6 Antibiotic Powders (Samara'a Drugs Company/ Iraq)

The following Antibiotic Powders used in the study:

Antibiotic	**code**
Ampicillin	AM
Amoxicillin+clavulanic acid	AMC
Amikacin	AK
Cefotaxime	CXT
Cephalexin	CL
Ciprofloxacin	CIP
Erythromycin	E
Gentamicin	CN
Naldixic acid	NA
Streptomycin	S
Tetracycline	TE
Trimetheprim+sulphamethoxazole	SXT

2.2 Methods

2.2.1 Solutions, Buffers and Reagents preparation

2.2.1.1 Antibiotic Solutions

a. Ampicillin, amikacin, tetracycline, streptomycin, aaldixic acid and cephalexin were prepared individually as stock solutions of 10 mg/ml of antibiotics powder in distilled water, sterilized by Millipore filter paper (0.2mm) and stored at -20°C (Maninatis *et al*., 1982).

b. Erythromycin was prepared as stock solution by dissolving 10 mg/ml of antibiotic powder in ethanol/water 50% (v/v) and sterilized by millipore filter paper (0.2mm) and stored at -20°C (Maninatis *et al*., 1982).

c. Cephotaxime was prepared as stock solution by dissolving 10 mg/ml of antibiotic powder in phosphate buffer pH 6.0 and sterilized by millipore filter paper (0.2mm) and stored at -20°C (Hawards and Jeffri, 1987).

d. Ciprofloxacin was prepared as stock solution by dissolving one gram of antibiotic powder in 90 ml sterile distilled water, pH was adjusted to 5.0 with 1 N HCl, then volume was completed to 100 ml, to obtain a final concentration of 10 mg/ml, sterilized by millipore filter paper (0.2mm) and stored at -20°C (AL-Yaseri, 1995).

2.2.1.2 Tris-EDTA Buffer

Stock Solution

Tris- EDTA solution was prepared by adding 1.861g of EDTA. $2H_2O$ and 6.055g of tris to 1000ml of distilled water, stirred vigorously by magnetic stirrer giving a final concentration of (5mM) of EDTA and (50mM) of Tris, than pH was adjusted to 8.0 with NaOH. The solution was dispensed into aliquots and sterilized by autoclave (Maninatis *et al*., 1982).

2.2.1.3 Ascorbic Acid Stock Solutions

Ascorbic acid solution was prepared by adding 10 g of ascorbic acid to 900 ml of sterile distilled water, pH was adjusted to 7.0 with 10 N NaOH, then the volume was completed to 1000 ml, giving a final concentration of 56.8 mM of ascorbic acid, and sterilized by Millipore filter paper (0.2mm) (Cursinon *et al*., 2005).

2.2.1.4 Phosphate Buffer pH 6.0 (Maniatis *et al*., 1982)

A/ Stock Solutions

a. 0.2 M solution of monobasic sodium phosphate: prepared by dissolving 31.2g NaH_2PO_4. $2H_2O$ in 1000 ml of distilled water.

b. 0.2 M solution of dibasic sodium phosphate: prepared by dissolving 28.39g of Na_2HPO_4 in 1000 ml of distilled water.

c. The buffer was prepared by mixing 87.7ml of solution a with 12.3ml of solution b and diluted to a total volume of 200 ml.

2.2.1.5 Reagents

a. Oxidase test reagent (Barom *et al*., 1994)

For this purpose, 1% solution was prepared by dissolving 1g of N, N, N, N, tetramethyl-p-phenyle diamine dihydrochlorid into 99 ml distilled water, then the volume was completed with distilled water to 100 ml.

b. Kovac's reagent (Colle *et al*., 1996)

It was prepared by dissolving 1g of para-dimethyl aminobenzaldehyde in 15 ml of isoamyl alcohol, then 5 ml of concentrated HCl was added carefully and gradually, then kept in refrigerator. This reagent was used in the indole test.

c. Catalase test reagent (Atlas *et al*., 1995)

A solution of 3% hydrogen peroxide (H_2O_2) was prepared by addition of 1 volume of 30% H_2O_2 to 9 volumes of sterile distilled water, and used for detecting the production of enzyme "catalase".

d. Gram stains solution:

Prepared as described by (Atlas *et al*., 1995).

2.2.2 Media Preparation:

a. Nutrient agar, Nutrient broth, MacConkey agar, and Muller Hinton agar:

These media were prepared as recommended by the manufacturing companies before autoclaving at 121º C for 15 minutes.

b. Blood agar:

It was prepared by autoclaving blood agar base at 121 ºC for 15 minutes, cooled to 50ºC; the human blood (Blood bank / Al- Kadhumia hospital) was added to give final concentration of 5%, mixed well and poured in Petri-dishes.

c. Chocolate agar:

It was prepared by autoclaving blood agar base at 121 ºC for 15 minutes, the human blood was immediately added to give final concentration of 5%, mixed well and poured in Petri-dishes.

d. Urea agar:

An amount of 950 ml of urea agar base (Christensen media) was prepared as recommended by manufacturing company, autoclaved at 121ºC for 15 minutes, cooled to 50ºC, then 50 ml of 40% urea (sterilized by Millipore filler (0.2)) was added and The medium was dispensed as slants.

e. Triple Sugar Iron agar (TSI) and Simone Citrate media:

These media were prepared as recommended by the manufacturing companies, before autoclaving at 121ºC for 15 minutes, then dispensed as slants.

2.2.3 Sterilization Methods:

i. Culture media (liquid and solid) and solutions were sterilized by autoclaving at 121ºC, 15 pounds / in² for 15 minutes.

ii. Glass wares were sterilized in the electric oven 180-200ºC for 2 hours.

iii. Thermo labile solutions such as, antibiotics solutions were sterilized by filtration through Millipore (0.2µm).

2.2.4 Specimens:

2.2.4.1 Specimen collection:

Sterile swabs were used for collection of Specimens from ear exudates of males and females with otitis media. All collected swabs were transported to the laboratory in an appropriate medium within a 30min.

2.2.4.2 Specimen culturing:

All specimens obtained were cultured directly and separately on the following solid media:

Blood agar

MacConkey agar

Chocolate agar

Swab was rolled across an area near the edge of the plate, then spreaded, streaked by a sterile loop, and incubated at 37ºC for 18-24 hours, except chocolate agar which was incubated anaerobically in a candle jar at 37ºC for 18-24 hours.

2.2.5 Identification of Bacteria

2.2.5.1 Direct smear

Direct smear was prepared on glass slide by mixing a small portion of the spacimen (colony) in 2 or 3 drops of water or saline, then a cover slip was applied and the prepared slide was examined microscopically. Gram stain was also used to identified gram positive and gram negative bacteria.

2.2.5.2 Biochemical Tests:

2.2.5.2.1 Gram positive bacteria

a. Mannitol salt agar (Baird, 1996).

It is a selective and indicator medium for the isolation of presumptive pathogenic Staphylococci. Most other bacteria are inhibited. It contains mannitol (1%) and sodium chloride (7.5%) with phenol red as indicator of acid production. *Staphylococcus aureus* and other staphylococci fermenting mannitol produce yellow colonies.

b. Deoxyribonuclease test (Cruickshank *et al*., 1975; Baird, 1996).

The DNase agar plate was divided into 6-8 sections by drawing lines and numbering the sections to denote the isolate .A colony from the primary culture was inoculated by spotting it on a small area of DNase agar in the middle of the marked section, then incubated at 37ºC for 18-24 hrs. Plate was flooded with few milliliters of (1N) HCl that precipitates the DNA and turns the plate cloudy. Appearance of clearing zone (absence turbidity) around the colony denotes DNase production and positive result.

c. Catalase test (Collee *et al*., 1996).

This test detects the presence of catalase enzyme, which produced by Staphylococci and most of Enterobacteriaceae. A small amount was picked from pure culture and put on a clean glass slide then one drop of 3% H_2O_2 was added, evolution of gas bubbles indicates a positive reaction.

d. Coagulase tests (Baird, 1996).

This test detects the presence of clumping factor in *Staphylococcus aureus*, where most of the other Staphylococci lacking. The tube method was used to detect this factor. After a Staphylococcus colony was let to grow in Brain-Heart broth for overnight at 37ºC, 0.1 ml of the growth culture was added to 0.5 ml of human blood plasma (Blood bank / Al-Kadhumia hospital) in a small tube (another tube was used as a control which contained human plasma only). The tubes were incubated at 37ºC for up-to four hours. A clot formation (by tilting the tube at 90º) was considered a positive reaction.

2.2.5.2.2 Gram negative bacteria

a. Oxidase test (Atlas, 1995).

The test was done by using a moisten filter paper with a few drops of a freshly prepared solution of N,N,N,N, tetra-methyl-p-phenylene diamine dihydro-chlorid, then a clump of cells was picked up aseptically from slant growth with a sterile wooden stick and smeared on the moistened paper, the development of violate or purple color within 10 seconds indicatespositive test.

b. Urease test (Atlas, 1995).

Urease activity was detected by inoculating the surface of Christensen urease agar slant with bacterial growth and incubated at 37ºC for 24 hrs, the appearance of red violate color indicates a positive test; whereas yellow-orange color indicates negative test.

c. Triple Sugar Iron (TSI) (Atlas, 1995).

Bacteria were cultured on TSI agar slant by stabbing and streaking on TSI slant surface then incubated for (24-48 hrs) at 37ºC. A color change in the medium from red to yellow this indicates acid formation (; and appearance of black precipitate indicates ferric sulfate formation; whereas pushing and cracking the agar to the top indicates CO_2 formation. All these results were positive.

d. Simon citrate test (collee *et al*., 1996).

Simon citrate agar was inoculated by streaking a loop full of bacterial growth on the surface of the medium, incubated at 37ºC for 24-48 hrs. Color changed from green to blue indicates positive result.

e. Indole test (Collee *et al*., 1996).

Five ml of peptone water was inoculated by bacterial colony, incubated at 37ºC for 48 hrs, then 0.05 ml of Kovac's reagent was added with mixing the contents of tube gently, appearance of red ring on the surface indicates positive result.

2.2.6 Identification of Gram Negative Bacteria by Api 20E

Identification of bacterial isolates was carried out by transferring single isolated colonies from MacConkey agar plates into Api 20E micro tubes system. This system is designed for the performance of more than

20 standard biochemical tests from a single colony on plating medium. Each test is preformed within sterile plastic micro tube which contains the appropriate substrate and is affixed to an impermeable plastic strip (gallery). Each gallery contains 20 micro tubes (which consists of a tube and a cupules section).

The biochemical tests included in this system are the following:

a. Beta-galactoside test ONPG.
b. Arginine dihydrolase test ADH.
c. Lysine decarboxylase test LDC.
d. Ornithine dearboxylase test ODC.
e. Citrate utilization test CIT.
f. Hydrogen sulphide test H_2S.
g. Urease test URE.
h. Tryptophane deaminase test TDA.
i. Indole test IND.
j. Voges proskauer test VP.
k. Gelatin liquefaction test GEL.
l. Glucose fermentation test GLU.
m. Mannitol fermentation test MAN.
n. Inositol fermentation test INO.
o. Sorbitol fermentation test SOR.
p. Rhamnose fermentation test RHA.
q. Sucrose fermentation test SAC.
r. Melibiose fermentation tests MEL.
s. Amygdalin fermentation tests AMY.
t. Arabinose fermentation test ARA.

The above 20 tests were preformed according to the manufacture instruction as follows:

i. Preparation of the galleries.

Five ml of tap water was dispensed into incubation tray to provide a humid atmosphere during incubation.

ii. Preparation of bacterial suspension.

A well-isolated colony was picked up by loop from MacConkey agar plate and was suspended in 5 ml sterile distilled water by rubbing against the side of the tube and mixed thoroughly with the water.

iii. Inoculation of galleries.

The twenty micro tubes were inoculated by a sterile pasture pipette, according to the manufactures instruction both the tube and the tube section of CIT, VP, and GEL micro tubes were filled.

After inoculation couple section of the ADH, LDC, ODC, H_2S, and URE micro tubes were completely filled with sterile mineral oil.

iv. Incubation of the galleries.

After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18 to 24 hrs at 37ºC.

v. Reading the galleries.

All the reactions not requiring reagents were recorded first, and then the following reagents were added to the corresponding micro tubes,

a. One drop of 3-4% ferric chloride to the TDA micro tube.

b. One drop of Kovac's reagent to the IND micro tube.

- (p-dimethyl aminobenzaldehyde at 4% in HCL isoamyl (alcohol)).

c. One drop of Voges-proskauer reagent to VP micro tube.

- Vp1 (40% potassium hydroxide).
- Vp2 (6% alpha-nephthal).

d. One drop of the oxidase reagent to ONPG micro tube.

The biochemical reactions preformed by the Api 20E and their interpretation are listed in table (2-1).

Table (2-1) Interpretation of reactions preformed by API 20E.

Micro-tube	Positive	Negative
ONPG	Yellow	Colorless
ADH	Red / Orange	Yellow
LDC	Orange	Yellow
ODC	Red / Orange	Yellow
CIT	Blue-green / Green	Pale green / Yellow
H_2S	Black deposit	Colorless / grayish
URE	Red / Orange	Yellow
TDA	Dark brown	Yellow
IND	Red ring	Yellow ring
VP	Pink / Red	Colorless
GEL	Diffusion of black pigment	No diffusion
GLU	Yellow	Blue / blue-green
MAN	Yellow	Blue / blue-green
INO	Yellow	Blue / blue-green
SOR	Yellow	Blue / blue-green
RHA	Yellow	Blue / blue-green
SAC	Yellow	Blue / blue-green
MEL	Yellow	Blue / blue-green

AMY	Yellow	Blue / blue-green
ARA	Yellow	Blue / blue-green

vi. Rapid identification at species and biotype level (Api supply)

Analytical profile index (numerical coding) was used. The biochemical profile obtained have to be transformed into numerical profile and to compare with those listed in the index by transforming all 20 biochemical results into seven-figures numerical profile (i.e., seven digit number) by placing them into group of three and consigning a specific value for each of the positive as follows:

Group 1			Group 2			Group 3			Group 4		
ONPG	ADH	LOC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU
1	2	4	1	2	4	1	2	4	1	2	

Group 5			Group 6			Group 7	
MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
1	2	4	1	2	4	1	2

Each positive reaction is given a value equal to 1, 2, or 4 according to the position of the test in its group. The sum of these values given the corresponding figure. Thus, the figure can have a value from 0 to 7 (0 for negative reaction). The seven digit numerical profile is then looked up in the index and the identification is determined.

2.2.7 Maintenance of Bacterial Isolates

Maintenance of bacterial isolates was preformed according to (Maniatis *et al*., 1982) as following:

a. Short term storage.

Bacterial isolates were maintained for short periods (2-3 weeks) on nutrient agar plate; the plates were tightly wrapped by parafilm (American National Can (U.S.A)) and stored at 4ºC.

b. Medium- term storage.

Bacterial isolates were maintained in nutrient agar stab culture for period of few months; such cultures were prepared in small screw-cup bottles containing (2-3) ml of nutrient agar media and stored at 4ºC.

c. Long term storage.

Cultures were stored freezing for long periods (1 year) in medium containing 15% glycerol. This availability was done by adding 1.5 ml of sterile glycerol to an exponential growth of bacteria in small screw-capped bottle with final volume 10 ml and stored at -20ºC.

2.2.8 Antibiotic Susceptibility Tests

A. Determination of Bacterial Susceptibility.

Disks diffusion method was used when 10 ml of nutrient broth was inoculated with part of the bacterial colony, and incubated at 37ºC for 18-24 hrs.

Bacterial culture (0.1 ml) was spreaded on the surface of Muller-Hinton agar plate by a sterile cotton swab in three different planes (by rotating the plate approximately 60º each time) to obtain an even distribution of inoculums throughout the plate. The inoculated plates were then placed at room temperature of 30 minutes to allow absorption of excess moisture. Then with sterile forceps, the selected antibiotics disks

were placed on the inoculated plates and incubated at 37ºC for 18 hrs in an inverted position.

After incubation, the diameters of inhibition zones were measured by a ruler. Results were compared according to the National Committee for Clinical Laboratory Standards (NCCL, 2001).

B. Determination of Minimum Inhibitory Concentration (MIC).

Minimum Inhibitory Concentration was determined using tubes dilution method in which each tube contained 10 ml of nutrient broth. After stock solution of antibiotic is prepared and sterilized by filtration, antibiotic was added to the first tube to give a final concentration of 1024 µg/ml and a final volume of 10 ml, then a serial dilutions were made by adding 5 ml from the first tube to 5 ml of the next tube and so on until the last tube. Each tube was then inoculated with 0.1 ml of previously prepared overnight culture of bacteria, and then the tubes were incubated at 37ºC for 24 hrs. Appearance of turbidity in the tubes and growth on Petri dishes indicates positive result.

2.2.9 Enhancement of Antibiotic Activity

a. Determining Effect of Tris-EDTA in Combination with Antibiotics

Different molarities of Tris-EDTA solution were prepared starting from (5mM of EDTA and 50mM of Tris). Lowest molarity of Tris-EDTA that causes inhibition against bacteria was determined using well diffusion assay.

After Tris-EDTA solution was prepared, the desired concentration of antibiotics was added. Paper disks were socked in the final solution, and disk diffusion assay was used to determine the inhibition zone of antibiotic disks against bacteria according to the National Committee for Clinical Laboratory Standards (NCCLs, 2001).

b. Determining Effect of Ascorbic acid in Combination with Antibiotics

Different molarities of ascorbic acid solution were prepared starting from (56.8mM), and the lowest molarities of ascorbic acid that causes inhibition against bacteria were determined using well diffusion assay.

Ascorbic acid solution was prepared and the desired concentration of antibiotics was added. paper disk was socked in the final solution, and disk diffusion assays were used to determine the inhibition zone of antibiotic disks against bacteria according to the National Committee for Clinical Laboratory Standards (NCCLs, 2001).

Chapter Three

Results And Discussion

Results and Discussion

3.1 Isolation and Identification of Microorganisms

3.1.1 Isolation

Eighty four swabs were collected from patients with otitis media attending Al-Kadhumia Educational Hospital and Central Public Health Laboratory of Ministry of Health during the period from 5/12/2006 to 28/5/2007.

After culturing of the 84 samples on media (MacConky agar, Blood agar, Chocolate agar), 54 samples gave positive bacterial growth, five gave fungal growth and 25 had no growth, this might due to treatment of patients with antibiotics.

3.1.2 Identification of Microorganisms

3.1.2.1 Morphologically.

Morphological characterization of the isolated microorganisms was carried out on the cultured media by the colonies formed. There were described according to the shape, size, color and other characteristics.

Pseudomonas areuginosa

Colonies of *Pseudomonas areuginosa* grown on MacConky agar medium appeared pale in color, with irregular edge, oval and large. On blood agar *Pseudomonas areuginosa* was able to hemolyse blood agar completely, while on nutrient and Muller Hinton agar, it produced blue-green pigment.

Microscopical examination of *Pseudomonas areuginosa* showed that the cells were gram negative, bacilli, appeared single, pairs or short chain and non spore forming. These results are comparable to the reported morphological characteristics of *Pseudomonas areuginosa*.

***Staphylococcus* species**

On blood agar, colonies of suspected *Staphylococcus aureus* appeared large, smooth, circular and pigmented (yellow or golden) surrounded by clear zone around the colonies and hemolysed blood completely. While *Staphylococcus epidermidis* appeared opaque, smooth and gray in color.

On mannitol salt agar, *Staphylococcus aureus* appeared yellow because of acid production from mannitol fermentation, while *Staphylococcus epidermidis* appeared pink because of non mannitol fermenting.

Microscopical examination of isolated both *Staphylococcus* species showed that they were gram positive, cocci, arranged in grape like clusters and nonspore forming.

Proteus mirabilis

On other hand, the isolates of *Proteus mirabilis* appeared pale, unable to ferment lactose on macConky agar and revealed their swarming motility on blood agar which characterized them from other *Enterobacterciea*.

Under the microscope, *Proteus mirabilis* cells appeared gram negative, bacilli and non pore forming.

Escherichia coli

Suspected colonies of *Escherichia coli* appeared glossy, pink and smooth with an entire edge on macConky agar.

Microscopical examination showed that suspected *Escherichia coli* cells were gram negative, short bacilli, non spore forming and occured singly.

3.1.2.2 Biochemical Tests

Further identification of the bacterial isolates was achieved by biochemical tests. The results which were shown in table (3-1) illustrated the biochemical characteristics of isolated gram negative bacteria.

Table (3-1) Biochemical characteristics of gram negative bacterial isolates.

Test \ Isolate		*Ps .aeruginosa*	*Pr. maribilis*	*E. coli*
TSI	Gas	+	+	+
	H_2S	−	+	−
	Reaction	Alk/No change	Alk/A	A/A
Urease		±	+	−
Citrate		+	+	−
Indole		−	−	+
Growth in 42°C		+	−	−
Oxidase		+	−	−
Catalase		+	+	+

+: positive result

−: negative result

±: variable result

Alk: alkaline

A: acid

Pseudomonas areuginosa gave positive results for citrate, oxidase, catalase and can grew at 42°C, while, it gave negative results for indole production, and variable result for urease production. *Pseudomonas areuginosa* produced alkaline slant (red) with no change bottom in test of sugar fermenting accompanied by gas production, but without black precipitate formation (no H_2S production).

Proteus maribilis gave positive results for urease, citrate and catalase, while, it gave negative results for indole production, oxidase and lactose fermentation. This species produced alkaline slant (red) with acid bottom

(yellow) in test of sugar fermenting accompanied by gas production and black precipitate formation (H_2S production).

Escherichia coli gave positive results for indole, catalase, while it gave negative result for urease production, citrate and oxidase. It produced acid slant (yellow) with acid bottom (yellow) in test of sugar fermenting accompanied by gas production, but without black precipitate formation (no H_2S production).

These results are similar to the results reported by Baron *et al*., 1994b; Holt *et al*., 1994 and Forbes *et al*., 2002.

The results reported in table (3-2) illustrated the biochemical characteristics of isolated gram positive bacterial isolates.

Table (3-2) Biochemical characteristics of gram positive bacterial isolates.

Isolate / Test	*Staph. aureus*	*Staph. epidermidis*
Oxidase	−	−
Catalase	+	+
Coagulase	+	−
Mannitol fermenter	+	−
DNase prodution	+	−

+: positive result

−: negative result

Staphylococcus aureus gave positive results for catalase, coagulase, mannitol fermenting ability and DNase production, but negative for oxidase. While *Staphylococcus epidermidis* gave negative results for oxidase, coagulase, mannitol fermenting ability and DNase production, whereas positive result was obtained for catalase.

These results are similar with the biochemical characteristics reported by Baron *et al*., 1994b; Holt *et al*., 1994 and Forbes *et al*., 2002.

Further identification of the isolates was done by using API 20E system for gram negative bacteria, (figure 3-1). These biochemical tests were confirmed by the diagnostic key (Mandelle *et al*., 1995).

A

B

C

Figure (3-1): Identification of gram negative bacteria by the API 20E system.

A: Biochemical Identification of *Proteus maribilius*.

B: Biochemical Identification of *E.coli.*

C: Biochemical Identification of *Pseudomonas aeruginosa.*

Numbers and percentages of the microorganisms isolated from the Otitis Media patients are shown in table 3-3. These results were obtained according to the for mentioned identification criteria of bacterial isolates.

Table (3-3) Numbers and percentages of microorganisms isolated from the Otitis Media patients.

Number of isolates	Types of Microorganisms	%
25	*Ps. aeruginosa*	29.76
3	*Pr. mirabilis*	3.58
5	*E. coli*	5.95
6	*Staph. aureus*	7.14
15	*Staph. epidermidis*	17.86
5	*Fungi*	5.95
25	No growth	29.76
84	-	100

Table (3-3) shows that 25 isolates of *Pseudomonas areuginosa* were obtained (29.76%), as the most common bacteria that caused chronic suppurative otitis media. Followed by 15 isolates of *Staphylococcus epidermidis* (17.86%), 6 of *Staphylococcus aureus* (7.14%), 5 of *Escherichia coli* (5.95%), 3 of *Proteus maribilis* (3.58%) and 5 of fungi (5.95%)

The obtained results were comparable to the results reported by Wariso and Ibe (2006) and Yeo *et al*. (2007) who mentioned that the most common bacteria of chronic suppurative otitis media was *Pseudomonas areuginosa*. This might be due to the resistance of these bacteria to most antibiotics, or to the production of extra-cellular enzymes, its exictance in the external ear and to its ability to enter middle ear through the perforation of tympanic membrane.

Other study Melaku and Lulseged, 1999) showed that the most common bacteria causing CSOM was *Proteus* 40 of 80 patients. They added

that is bacteria can enter the external ear and causes infection when the resistance of middle ear is lowered.

While Jha *et al*. (2007) showed in his study that the more common bacteria causing ear discharge was *Staphylococcus aureus* (34.3%). This may be due to inherent nature of bacteria to develop resistance strains and ability to enter the middle ear through the external canal as normal flora (Blustone and Stool, 1983).

3.2 Age Distribution

A total of 54 patients who infected with bacterial infection were between the ages 1-60 years were enrolled in this study, they were divided into six groups.

Results of table (3-4) indicate that the majority (46.3%) of patients belonged to the age group of 1-10 years. Mishra *et al*. (1997) who found that 66.67% cases of chronic supprative otitis media were detected in the age group of less than 14 years. This declares that children were more prone to CSOM than adults.

High incidence of otitis media in children may be related to the Eustachian tube which is shorter, more horizontal and more compliant than that of older person (Roddey and Hoover, 2000), nutritional deficiencies, lack of hygiene and other features.

Table (3-4) Distribution of chronic suppurative otitis media according to patient age groups.

Age (year)	No. of cases	%
1-10	25	46.3
11-20	13	24.1
21-30	9	16.7
31-40	4	7.4
41-50	1	1.8
51-60	2	3.7
Total	54	100

3.3 Sex Distribution

It was found in this study that occurrence of the positive bacterial agents was higher in male (57.4%) than female (42.6) (table 3-5).

Table (3-5) Distribution of chronic suppurative otitis media according to the sex of patients.

sex	Number of positive cases	%
male	31	57.4
Female	23	42.6
Total	54	100

Marchant *et al.* (1986) and Kaleida *et al*. (1991) reported that the incidence of male was approximately 53%. On other hand, Del Beccaro *et al.* (1992) found the opposite when they reported that incidence in female 60% was higher than male 40%. Other study showed equal distribution between male and female (Loy *et al*., 2002).

Our results came in agreement with several investigators. Obi *et al*. (1995) and Wariso and Ibe (2006) found that male infected with otitis media more than female.

Male predominance may be due to more exposed to ways of life. These differences in both sexes may also due to presumably reflecting sex-related differences, child care-practice and low levels of socio-economic status.

3.4 Ear's Site of Infection

Results shown in table (3-6) demonstrated that infection of the right ear was slightly higher than the left and both ears (50%, 45.4% and 5.6%) respectively.

Table (3-6) Numbers and percentages of ear's site of infection (right, left or both).

Site of infection	Number	%
Right	27	50
Left	24	45.4
Both	3	5.6
Total	54	100

Similar results were reported by Ferede *et al*. (2001) when they found that the right ear infections were more common than the left and both ears, they were 50(44%), 38(34%) and 24(22%), respectively.

While Kamal *et al*. (2004) showed different results, which revealed that the incidence of left ear infections was more than the right and both (46.7%, 26.7% and 26.7%) respectively.

High incidence of right ear infection may be due to attributed to that most children are right handed, and might scratch their right ear.

Table (3-7) showed the distribution of ear's site infections in OM patients in both sexes. Results demonstrated that the male's infections were 26% for the right ear, 27.8% for left ear and 3.7% for both ears. Percentages of female's infections were 24% for right ear, 16.7% for left ear and 1.9% for both ears.

Table (3-7) Numbers and percentages of ear's site OM infections between both sexes.

Sex	Right ear		Left ear		Both ears		Total	
	No.	%	No.	%	No.	%	No.	%
Male	14	26	15	27.8	2	3.7	31	57.4
Female	13	24	9	16.7	1	1.9	23	42.6
Total	27	50	24	44.5	3	5.6	54	100

It is possible to observe in the same table that the right and the left ear's infections were slightly increased in males (26%, 27.8% respectively) than females (24%, 16.7% respectively), infections in both ear's sites were lower, in male 3.7% and female 1.9%.

3.5 Distribution of Otitis Media according to Months

The results reported in our study showed that the incidence of OM infections was higher in February than other months. Table (3.8) showed the variation in monthly distribution of OM patients.

Table (3-8) Numbers and percentages OM patients distributed according to the month of year.

Month	Number of patients	%
December	13	24.1
January	11	20.3
February	18	33.3
March	7	13
April	5	9.3
May	0	0
Total	54	100

Winter's months (December, January and February) had highest incidence of OM infection (24.1%, 20.3% and 33.3% respectively). The incidences declined in the following months, for example in March and April they were 13% and 9.3% respectively; while in May no bacterial infection was obtained in this study. These results were closed to those obtained by Green and Rothrock (1993) who found that the highest incidence of patients was in winter season (48%), which declined in spring (16%) and summer (4%). In this respect, Guevara *et al*. (2008) who showed that the bacterial infections detected during the rainy season was higher than during the dry season.

Engel *et al*. (1999) reported that season appeared to have a significant effect on OM infections. It may be attributed to high incidence of viral URT infection which leads to secondary bacterial infection causing OM (Byington, 1998).

3.6 Distribution of Single and Mixed Infections of Patients

Table (3.9) shows that single infection was more frequent than the mixed infection. The incidence of single infection was (90.7%), whereas for mixed infections was (9.3%). Dincer, *et al*. (1992) showed that the pure infection was 58.8% in comparison with the mixed (35.3%). Other investigators observed similar results (Anifasi and Tumushime, 1989; Aslam, *et al*., 2005; Jha, *et al*., 2007).

Table (3-9) Numbers and percentages of single and mixed infections of patients.

Bacteria	Single infection	Mixed infection	Total	%
P. aeruginosa	24	1	25	46.3
P. mirabilis	2	1	3	5.6
E. coli	3	2	5	9.3
Staph.aureus	6	_	6	11
Staph.epidermidis	14	1	15	27.8
Total	49	5	54	100

3.7 Antibiotic Sensitivity

Testing antibiotics resistance in microorganism is important for categorizing their behavior in accordance with the kinds of antibiotics as well as their medical application and effectiveness in treatment of disease. Moreover, it will serve to give a picture to the following transfer of genetic elements responsible for resistance among species and hence detection of spread of resistance (Chambers, 2001).

For screening purposes, the required test should be suitable for large number of isolates. Disk diffusion method (which is used in this study) is fast

and simple; but, is with lower accuracy as long as zone of inhibition is widely affected by medium composition and interference of some ions with spread of antibiotics through the medium (Baron *et al*., 1994).

For research studies, minimum inhibitory concentration (MIC) is more useful to specify minimum concentration of antibiotics showing inhibition of growth. Concentration for example could also be useful for medical purposes. Accordingly, for testing antibiotics sensitivity, the two methods were used wherever it is needed (Nester, *et al*., 2001).

3.7.1 Disk Diffusion Test.

The emergence and prevalence of antibiotic resistant strains are considered as a major therapeutic problem that can be explained by several hypotheses such as the influence of excessive and/or misuse of antibiotic use (Sotto *et al*., 2001).

Standard disk diffusion assay was used to detect the sensitivity of pathogenic bacteria and results obtained were compared with those of (NCCLs, 2001).

Bacterial (54) isolates were screened for their susceptibility to twelve antibiotics representing different groups. Results which are shown in table (3-10) indicate variable resistance profiles among isolates against the antibiotics used.

Table (3-10) Antibiotics susceptibility for different isolates from CSOM.

Antibiotics / Isolates	CL	CIP	S	AMC	AK	CTX	TE	E	AM	SXT	CN	NA
***Ps.* 1**	R	R	R	R	R	R	R	R	R	R	R	R
***Ps.* 2**	R	S	R	S	R	S	R	R	R	R	R	S
***Ps.* 3**	R	S	R	R	S	S	S	R	R	S	S	S
***Ps.* 4**	R	S	R	R	R	R	R	R	R	S	R	S
***Ps.* 5**	R	S	R	R	R	S	R	R	R	S	R	R
***Ps.* 6**	R	S	R	R	R	R	R	R	R	R	R	R
***Ps.* 7**	R	R	R	R	S	R	R	R	R	R	R	R
***Ps.* 8**	R	R	S	R	S	R	R	R	R	R	S	R
***Ps.* 9**	R	S	R	S	S	S	R	R	R	S	S	R
***Ps.* 10**	R	S	R	R	R	R	R	R	R	R	R	R
***Ps.* 11**	R	S	R	S	S	S	R	R	R	S	S	R
***Ps.* 12**	R	S	R	R	R	R	R	R	R	R	R	R
***Ps.* 13**	R	S	R	R	R	R	R	R	R	R	R	R
***Ps.* 14**	R	S	R	R	S	R	R	R	R	S	S	R
***Ps.* 15**	R	S	R	R	S	R	R	R	R	R	R	S
***Ps.* 16**	R	S	R	R	R	R	R	R	R	R	R	R
***Ps.* 17**	R	S	R	R	S	R	R	R	R	S	S	R
***Ps.* 18**	R	S	R	R	S	R	R	R	R	R	R	R
***Ps.* 19**	R	S	R	R	S	R	R	R	R	R	R	R
***Ps.* 20**	R	S	R	R	S	R	R	R	R	R	R	R
***Ps.* 21**	R	S	S	R	S	R	R	R	R	S	S	R
***Ps.* 22**	R	S	R	R	R	R	R	R	R	R	R	R
***Ps.* 23**	R	S	R	R	S	R	R	R	R	R	S	R
***Ps.* 24**	R	R	R	R	R	R	R	R	R	R	R	R
***Ps.* 25**	R	S	R	R	R	R	R	R	R	R	R	R
***Pr.* 26**	R	S	S	S	S	R	R	R	R	S	S	S
***Pr.* 27**	R	S	S	S	S	R	R	R	R	S	S	S
***Pr.* 28**	R	S	S	R	S	S	R	R	R	R	S	S
***E. coli* 29**	R	S	R	R	S	R	R	R	R	S	S	S
***E. coli* 30**	R	R	R	R	S	R	R	R	R	R	R	R
***E. coli* 31**	R	S	R	R	S	R	S	R	R	S	R	S
***E. coli* 32**	R	S	R	R	S	R	R	R	R	S	S	S
***E. coli* 33**	R	S	R	R	S	R	R	R	R	R	S	R
***Staph. aureus*34**	R	S	S	R	S	R	S	R	R	R	S	R
***Staph. aureus*35**	R	S	S	R	S	R	R	R	R	S	S	S
***Staph. aureus*36**	R	S	R	R	R	R	S	S	R	S	S	R
***Staph. aureus*37**	R	S	R	R	R	R	S	S	R	S	R	R
***Staph. aureus*38**	R	S	R	R	R	R	R	R	R	R	R	S

***Staph. aureus*39**	**R**	**S**	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**S**	**S**	**R**
***Staph. epidermidis*40**	**R**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**	**S**
***Staph. epidermidis*41**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**S**	**S**	**S**
***Staph. epidermidis*42**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**
***Staph. epidermidis*43**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
***Staph. epidermidis*44**	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**R**
***Staph. epidermidis*45**	**R**	**S**	**R**	**S**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**
***Staph. epidermidis*46**	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**S**	**R**
***Staph. epidermidis*47**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**S**	**S**
***Staph. epidermidis*48**	**R**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**S**
***Staph. epidermidis*49**	**R**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**S**	**S**
***Staph. epidermidis*50**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**S**	**R**
***Staph. epidermidis*51**	**R**	**S**	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**R**	**R**
***Staph. epidermidis*52**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
***Staph. epidermidis*53**	**R**	**S**	**R**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**S**	**R**
***Staph. epidermidis*54**	**R**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**R**

S: Sensetive, **R**:Resistance, ***Ps.***: *Pseudomonas aeruginosa*, ***Pr.***: *Proteus mirabilis*, ***Staph. aureus***: Staph. aurous, ***Staph. epidermidis***: *Staph. epidermidis*, ***E. coli***: *E. coli.*
CL: Cephalexin, **CIP**: Ciprofloxacin, **S**: Streptomycin, **AMC**: Augmentin, **AK**: Amikacin, **CTX**: Cefotaxim, **TE**: Tetracyclin, **E**: Erythromycin, **AM**: Ampicillin, **SXT**: Trimetheprim+sulphamethoxazole, **CN**: Gentamycin, **NA**: Nalidixic acid.

Figure (3-2) shows that resistance of gram negative bacterial isolates to the tested antibiotics were 100% to each of cephalexin and erythromycin, 93.9% for tetracycline, 90.4% for amoxicillin, but lower resistance to amikacin (36.3%) and ciprofloxacin (15.1%). Others were distributed in between.

On other hand, same figure showes that the antibiotic resistance of gram positive bacteria were 100% for cephalexin and ampicillin, 66.6% for erythromycin and nalidixic acid, and 61.4% for streptomycin and Amoxicillin+clavulanic acid (augmentin), whereas the others distributed in between.

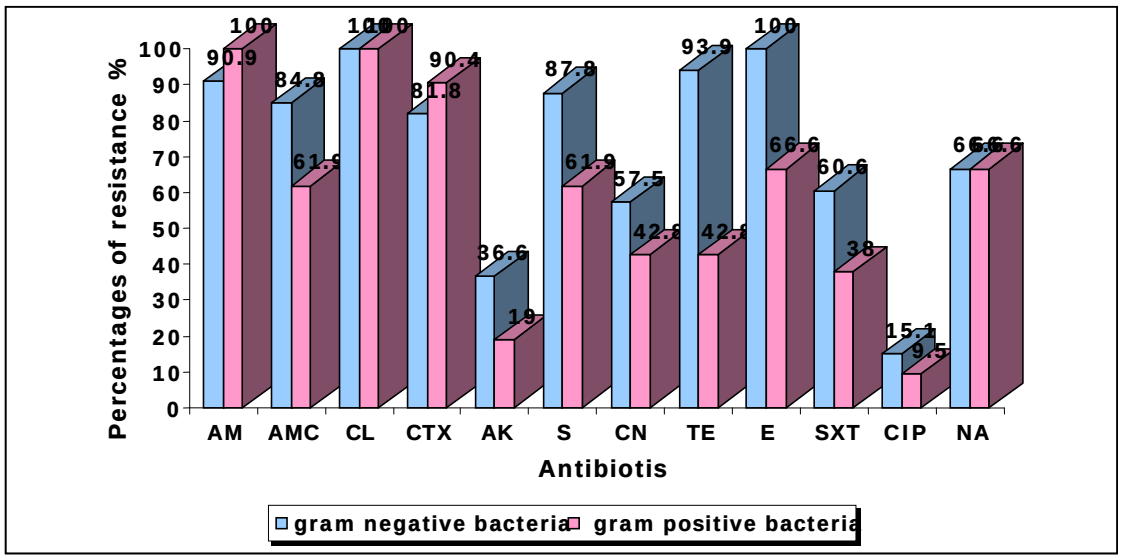


Figure (3-2) Percentages of antibiotics resistance of gram negative and positive bacteria.

In this study, isolates showed highest resistance to ampicillin (100%) in all types of bacteria. This result agree with the results obtained by Compose *et al*. (1995) who found that *Ps. aeruginosa*, *Pr. mirabilis* and *Staph.aureus* were completely resistant to ampicillin (100%), while Moshi *et al*. (2000) revealed that *Ps. aeruginosa* was resistant to ampicillin at 89.9%. Other study carried out by Ountibeju (2003) showed that *Pr. mirabilis*, *Staph.aureus* and *E. coli* were resistant to ampicillin at 72.7%, 50%, 50% respectively.

Table (3-11) Percentages of antibiotics resistance bacteria tested.

Bacteria / antibiotics	*Ps.*	*E. coli*	*Pr.*	*Staph.aureu*	*Staph.epid*
AM	100	100	100	100	100
AMC	88	100	33.3	100	46.6
CL	100	100	100	100	100
CTX	80	100	66.6	100	80
AK	48	0	0	66.6	0
S	92	100	33.3	66.6	60
CN	72	40	0	33.3	46.6
TE	96	80	100	33.3	46.6
E	100	100	100	50	73.3
SXT	64	40	33.3	33.3	40
CIP	16	20	0	0	6.6
NA	68	40	0	66.6	66.6

Ps*: Ps. aeruginosa*, ***Pr:*** *Pr. mirabilis*, ***Staph.aureu:*** *Staph.aureus*, ***Staph.epid***: *Staph.epi dermidis*. **CL:** Cephalexin, **CIP**: Ciprofloxacin, **S**: Streptomycin, **AMC**: Augmentin, **AK**: Amikacin, **CTX**: Cefotaxim, **TE**: Tetracyclin, **E**: Erythromycin, **AM**: Ampicillin, **SXT**: Trimetheprim+sulphamethoxazole, **CN**: Gentamycin, **NA**: Nalidixic acid.

The results reported in table (3-11) demonstrated that *Staph.aureus* and *E. coli* showed high resistance to augmentin (100%), while *Ps. aeruginosa, Pr. mirabilis* and *Staph.epidermidis* showed 88%, 33.3%, 46.6% respectively. Ferede *et al*. (2001) reported different results; they showed lower resistance of *Pr. mirabilis*, *Staph.aureus* and *E. coli* to augmentin 5.8%, 17.8%, 15.3% respectively, while Gül *et al*. (2006) indicated that *Ps. aeruginosa* had high resistance to augmentin (85%).

Resistance to augmentin (combination of β-lacame/β-lacamase inhibitor) combination may be due to the overproduction of β-lacamase by the resistant isolates which overcome the β-lacame inhibitor action (Chambers *et al*., 2001).

The study also showed high resistance to cephalexin when all types of bacterial isolates were resistant to it (100%). Sensitivity of *Staph.aureus* to cephalexin was reported by Loy *et al*. (2002) while Poorey and Layer (2002) found that the gram negative bacteria (*Ps. aeruginosa* 88.9%, *Pr. mirabilis* and *E. coli* 50%) were more resistant than the gram positive bacteria (*Staph.aureus* 26.7% and 100% sensitive for *Staph.epidermidis*).

The reported results in this study also showed that the resistance to cefotaxime was high for all isolated bacteria; *E. coli* and *Staph.aureus* (100%) *Ps. aeruginosa* and *Staph.epidermidis* (80%), and 66.6% for *Pr. mirabilis*. The obtained results disagree with Poorey and Layer (2002) who found lower resistance for all tested bacteria in their investigation were *Ps. aeruginosa* (27.2%), *E. coli* (33.4%), *Staph.aureus* (20%), *Staph.epidermidis* (40%) and (100%) sensitive *Pr. mirabilis*. Aslam *et al*. (2005) showed that *Ps. aeruginosa* was resistant to cefotaxime at 58.4%.

The reason for high resistance percentages by bacterial isolates to β-lacame antibiotics may be due to the wide and unwise use of antibiotics which leads may develop the resistance through the action of β-lacamase enzymes. (Rodrigues *et al*., 2004).

Resistance to third generation of cephalosporin (cefotaxime) was mainly due to ESBL enzymes that can hydrolyze these antibiotics (Babypadmini and Appalaraji, 2003).

The reported resistance to aminoglycosides in this study was found to be variable. amikacin was the most effective one against different isolated bacteria; 48% resistance was shown by *Ps. aeruginosa*. This was close to that

found by Jang and Park (2004) when reported the resistant of *Ps. aeruginosa* was 44.4%. On the other side, *Pr. mirabilis*, *E. coli* and *Staph. epidermidis* were 100% sensitive to the amikacin; whereas 66.6% resistance was observed for *Staph. aureus*. Another results were observed by Poorey and Layer (2002) who found that *Staph. epidermidis* (40%) sensitivitivity to amikacin and *Staph. aureus* (33.4%) resistance to this antibiotic.

Regarding streptomycin, all bacterial isolates showed high resistance percentages to it, except *Pr. mirabilis* that had low resistance. *Ps. aeruginosa*, *E.coli*, *Staph. aureus* and *Staph. epidermidis* had resistance 92%, 100%, 66.6%, 60% respectively, while *Pr. mirabilis* 33.3%. These results disagreed with those of Ferede *et al*. (2001) who reported high resistance to *Pr. mirabilis*, *Staph. aureus*, *E. coli* and *Ps. aeruginosa* (47%, 42.8%, 53.6%, 90%, respectively).

Variable resistances to gentamycin by different isolated bacteria were recorded. Percentages of resistance were 72%, 40%, 33.3%.46.6% to *Ps. aeruginosa, E. coli*, *Staph. aureus* and *Staph. epidermidis*, respectively. *Pr. mirabilis* isolates were 100% sensitive. Aslam *et al*. (2005) and Gul *et al*. (2007) reported gentamycin sensitivity for *Ps. aeruginosa* 40%, 30% respectively. While Poorey and Layer (2002) found similar results to that obtained in this study, when they reported 100% of gentamycin sensitivity for *Pr. mirabilis*, and 60% for *Staph. epidermidis*.

Oguntibeju (2003) showed that gentamucin resistance to *E. coli* was 20%; other study by Gül *et al*. (2006) found 27% gentamucin resistance to *Staph. aureus*.

All isolated bacteria could resist aminoglycoside by producting modifying enzymes affecting the aminoglycosides and prevent them from binding to ribosome (Jawetz *et al*., 1998).

Quinolones on the other hand had shown good effect on the bacterial isolates. Most isolates found to be sensitive; for example ciprofloxacin sensitivity was 100% for each of *Pr. mirabilis* and *Staph. aureus*, 80% for *E. coli*, 84% for *Ps. aeruginosa* and 93.4% for *Staph. epidermidis*. These results were almost comparable to the results reported by Poorey and Layer (2002) who found that ciprofloxacin sensitivity of *Pr. mirabilis*, *Ps. aeruginosa* and *Staph. epidermidis* were100%, 86%, 80% respectively, while for *E. coli* was 16.6% . Indudharan *et al*. (1999) detected 98.7% ciprofloxacin sensitivity of *Staph. aureus.*

Sensitivity of bacteria to ciprofloxacin (quinolones) because quinolones act principally by inhibiting bacterial DNA Gyrase, so preventing supercoling of the DNA; a process that is necessary to compacting chromosome into the bacterial cell.

While nalidixic acid (other member of quinolones) showed different resistant to bacteria; 68% for *Ps. aeruginosa*, 66.6% for (*Staph. aureus* and *Staph. epidermidis*), and 40% for *E. coli* while 100% sensitive to *Pr. mirabilis*.

Resistance of bacteria to nalidixic acid may be due to the development of resistant gene carried by conjugative plasmid (Martinez-martinez *et al*., 1998).

Results showed high resistance to erythromycin with 100% for each of (*Ps. aeruginosa, Pr. maribilus* and *E. coli*), 73.3% for *Staph. aureus* and 50% for *Staph. epidermidis*. Oguntibeju (2003) found low resistance to *Staph. aureus* and *E. coli* (10%). While Poorey and Layer (2002) mentioned that *Ps. aeruginosa* resistance to erythromycin was 69.5%.

Resistance to erythromycin may be due to the common use of this antibiotic which leads to increase microbial resistance to this antibiotic.

Tetracycline also showed high resistance to *Ps. aeruginosa* 96%, *Pr. mirabilis* 100%, *E. coli* 80%, *Staph. epidermidis* 46.6% and 33.3% for *Staph. aureus*. Moshi *et al*. (2000) showed high resistance to tetracycline, 91.7% for *Ps. aeruginosa* and Ferede *et al*. (2001) also showed high resistance of *Pr. mirabilis* 76.4%, *E. coli* 84.6%, *Ps. aeruginosa* 100% and 78% for *Staph. aureus*.

Resistance to Tetracycline may be due to the resistance gene which carried by plasmid (Merlin *et al*., 1988).

Resistance to Trimetheprim-sulphamethoxazole SXT were 64% for *Ps. aeruginosa*, 40% for *E. coli* and *Staph. epidermidis* and 33.3 for *Staph. aureus* and *Pr. mirabilis*. Gül *et al*. (2006) showed in closed result, regarding SXT (37% for *Staph. aureus*). Park *et al*. (2008) showed different when SXT resistance to *Staph. aureus* was 11.8%; while ferede *et al*.(2001) found that the SXT resistance to *E. coli*, *Pr. mirabilis* and *Ps. aeruginosa* 38.4%, 23.5%, 100%, respectively.

The results in table (3-10) showed that nine isolates (*Ps* 1, *Ps* 6, *Ps* 15, *Ps* 19, *Pr* 28, *E.coli* 30, *Staph. aurous* 38, *Staph. epidermidis* 43, *Staph. epidermidis* 51) possessed the highest level of resistance, so that they were selected to investigate the minimum inhibitory concentration of antibiotics against selected bacteria.

3.7.2 Minimum Inhibitory Concentration (MIC)

The nine selected isolates which have the highest level of resistance were tested to determine the MIC of ten antibiotics; Ampicillin, Cephalexin, and Cefotaxime (β-lactams), Amikacin, Gentamicin and Streptomycin (aminoglycosides), Ciprofloxacin and Naldixic acid (quinolons), Tetracycline and Erythromycin as in table (3-12).

The breakpoints were applied following NCCLs (2001) recommendation. When resistance levels were calculated, "MIC" in both the intermediate and resistance range (as defined by the NCCLs 2001) considered as non susceptible in accordance with the definition of MIC as the lowest drug concentration in microgram per milliliter that inhibited the visible growth of the bacteria (Kinoshita *et al*., 1997). It was concluded that Ciprofloxacin had the extended spectrum of antibacterial activity, a result which agreed with that obtained by Ettehad *et al*. (2006).

Table (3-12) MIC's of ten antibiotics against nine selected isolates.

Isolate	MIC									
	CN	CIP	S	AK	TE	E	CL	CTX	AM	NA
Ps 1	128	8	128	128	128	256	128	128	1024	128
Ps 6	128	4	128	128	64	512	156	512	1024	64
Ps 15	32	2	32	8	256	128	512	256	1024	512
Ps 19	64	8	64	8	32	256	256	512	1024	256
Pr 28	8	2	32	4	256	512	512	16	512	16
E.coli 30	32	8	128	16	64	128	128	256	1024	256
Staph. aureus 38	64	2	32	128	16	32	1024	512	1024	512
Staph. epidermidis 43	32	8	64	2	32	256	1024	128	512	128
Staph. epidermidis 51	128	2	128	4	64	64	128	64	1024	64

CL: Cephalexin, **CIP**: Ciprofloxacin, **S**: Streptomycin, **AK**: Amikacin, **CTX**: Cefotaxim, **TE**: Tetracyclin, **E**: Erythromycin, **AM**: Ampicillin, **CN**: Gentamycin, **NA**: Nalidixic acid.
Ps**:** *Pseudomonas aeruginosa*, ***Pr:*** *Proteus mirabilis*, ***Staph. aureus*****:** *Staphylococcus aureus*, ***Staph. epidermidis*****:** , ***E.coli*****:** *E. coli*.

All isolates gave high resistance to tetracyclin, erythromycin, cephalexin, cefotaxim, ampicillin and nalidixic acid, with MIC's of (16-256), (32-512), (128-1024), (16-512), (512-1024), (16-512) µg/ml respectively. This is may be due to wide and unwise use of antibiotics. These results were comparable with the results obtained by Qadri *et al*. (1994) who showed in their study that isolated bacteria gave high resistance to β-lactam antibiotics. The obtained results showed that all tested bacteria were less resistance to aminoglycosides (amikacin 2-128, gentamycin 8-128, streptomycin 32-128). On the other hand, the tested bacteria exhibited very low resistance to Ciprofloxacin (2-8 µg/ml).

Antonio-Velmonte *et al*. (1986) showed that the aminoglycoside's MICs for *E. coli* and *Proteus mirabilis* were in rang between (0.25-256) µg/ml, while Gesu *et al*. (2003) recorded higher ciprofloxacin MIC ranged (32-64µg/ml). Rashedko *et al*. (2002) found that MIC's of aminoglycosides for *Pseudomonas aeruginosa* were (0.25-512) µg/ml.

3.8 Enhancement of Antibiotic Activity

3.8.1 Effect of Tris-EDTA in Combination with Antibiotics.

Results presented in table (3-13) and figure (3-3) showed the relation between Tris-EDTA and different antibiotics used. Tris-EDTA and *B*-lactam antibiotics showed synergistic effect against tested isolates of gram negative and gram positive bacteria, also tetracycline showed synergistic effect on ***Pr*28** (*Proteus mirabilis*) isolate when used with Tris-EDTA. These results were comparable with Vaara (1992) who reported that EDTA combination with penicillin and tetracycline caused reduction of MIC, also Farca *et al*. (1994) who showed in their study that Tris-EDTA potentiated the activity of β-lactam antibiotics.

Table (3-13) Effect of combination of Tris-EDTA with antibiotics against different types of bacteria using disk diffusion assay.

Isolate	Antibiotic	Inhibition zone diameter (mm)	
		Antibiotics alone	Antibiotics and Tris-EDTA
Ps 1	CIP	13	10
	S	10	11
	AK	14	15
	CTX	0	12
	CN	9	12
Ps 6	CIP	27	17
	AK	0	10
Ps 15	CIP	23	12
	AK	17	19
	CTX	0	13
	CN	0	9
Ps 19	CL	0	9
	CIP	27	11
	S	0	11
	CTX	0	13
	E	0	10
	AM	0	12
	SXT	0	9
	CN	0	10
	NA	0	11
Pr 28	CL	0	10
	CIP	40	0
	AMC	0	13
	CTX	20	0
	TE	0	12
	CN	15	0
	NA	20	0

E. coli 30	SXT	0	10
	CN	10	13
Staph. aureus 38	CL	0	25
	CIP	20	25
	S	10	17
	AMC	0	20
	AK	12	27
	CTX	0	18
	TE	18	25
	E	14	23
	AM	0	13
	SXT	19	23
	CN	12	21
	NA	0	20
Staph. epidermidis 43	CIP	0	19
	AMC	0	11
	AK	20	18
	CTX	0	14
	TE	0	12
	CN	0	16
	NA	0	14
Staph. epidermidis 51	CIP	22	28
	AMC	20	22
	AK	17	20
	CTX	0	15
	TE	0	11
	SXT	18	20
	CN	0	13
	NA	0	1

***Ps*: *Pseudomonas aeruginosa*, ***Pr*: *Proteus mirabilis*, ***Staph. aureus*:** *Staphylococcus aureus*, ***Staph. epidermidis*:** *Staphylococcus epidermidis*

***E. coli*:** *Escherichia coli*

On the other hand, antagonistic effect between Tris-EDTA and quinolone was observed by all tested gram negative bacteria, while, ***Ps* 19** (*Pseudomonas aeruginosa*); showed synergistic effect with nalidixic acid, these results agreed with Amara and Hussein (2006) who showed that antagonistic effect between Tris-EDTA and ciprofloxacin against *Pseudomonas aeruginosa*, while Gotthelf (2003) reported that EDTA was capable of reducing the MIC of ciprofloxacin against *Pseudomonas aeruginosa*.

Adversely, synergestic effect between Tris-EDTA and quinolone was observed aginst all tested gram positive bacteria.

Synergistic effect between Tris-EDTA and aminoglycosides was observed against tested bacteria; antagonistic effect was shown against ***Pr*28** (*Proteus mirabilis*). These results agreed with Spark, *et al*. (1994) and Foster and DeBore (1998) who showed synergistic effect when combination of Tris-EDTA and aminoglycosides were used against *Pseudomonas aeruginosa and E. coli.* Farca *et al*., (1994) showed in their study that Tris-EDTA potentiated the activity of streptomycin by binding to the metal ions which compete with aminoglcoside antibiotics for cell wall receptors that allow antibiotics to enter the bacterial cell.

Synergetic effect between SXT antibiotics and Tris-EDTA was shown against Gram negative bacteria, while erythromycin and Tris-EDTA gave synergetic effect against it.

The synergistic effect between Tris-EDTA and antibiotics may be due to the remove of Mg^{+2}, Ca^{+2} from outer membrane of the bacterial cells sites, leading to the release of cell wall lipopolysacchrides, protein and other cell contents of the cell wall (Nicas and Hancock, 1983 and Foster and DeBore, 1998). Another reason may be due to Tris-EDTA enhancement of antibiotics activity by binding to the metal ions which compete with antibiotics for cell

wall receptors allowing antibiotics to enter the bacterial cell (Nicas and Hancock, 1983).

3.8.2 Effect of Ascorbic acid in Combination with Antibiotics.

Results presented in table (3-14) and figure (3-3) showed the effects of combination of ascorbic acid and various antibiotics against test bacteria.

Synergistic effect between β-lactam antibiotics and ascorbic acid was observed against gram negative and positive bacteria. Whereas antagonistic effect between quinolones and ascorbic acid was shown against gram negative bacteria except ***Ps*19** isolate of (*Pseudomonas aeruginosa*) which was showed synergestic effect with nalidixic acid.

Moreover, a synergistic effect between quinolones and ascorbic acid was noticed against gram positive bacteria.

Synergistic effect between aminoglycoside and ascorbic acid was shown against gram negative and positive bacteria ***Pr*28** (*Proteus mirabilis*).

Erythromycin and SXT showed synergistic effect with ascorbic acid toward ***Ps*19** (*Pseudomonas aeruginosa*), also tetracycline showed synergistic effect with ascorbic acid against R28 (*Proteus mirabilis*).

The synergistic effect of ascorbic acid with antibiotics may be due to the effect of ascorbic acid on some metabolic activity associated with protein synthesis inside the bacterial cells and tendering the organisms more permeable to antibiotics through the effect on cell membrane and providing easier and larger penetration of antibiotics to inside the cell, or may be due to the effect of H_2O_2 that released from auto-oxidation of ascorbic acid that lead to potent the activity of antibiotics (Kramarenko *et al*., 2007).

Other studies have shown that some antibiotics stimulate the induction of reactive oxygen species (free radical) in different bacterial species (Becerra and Albesa, 2002; Albesa *et al*., 2004), so may be ascorbic acid acts as

scavengers for this free radical. On other hand, the loss of antibiotics resistance in *Staphylococci* might due to the possible plasmid curing effect of ascorbic acid (acts as curing agents) that lead to loss of plasmid (Amabile-Cuevas, 1988). This explains the antagonistic effect.

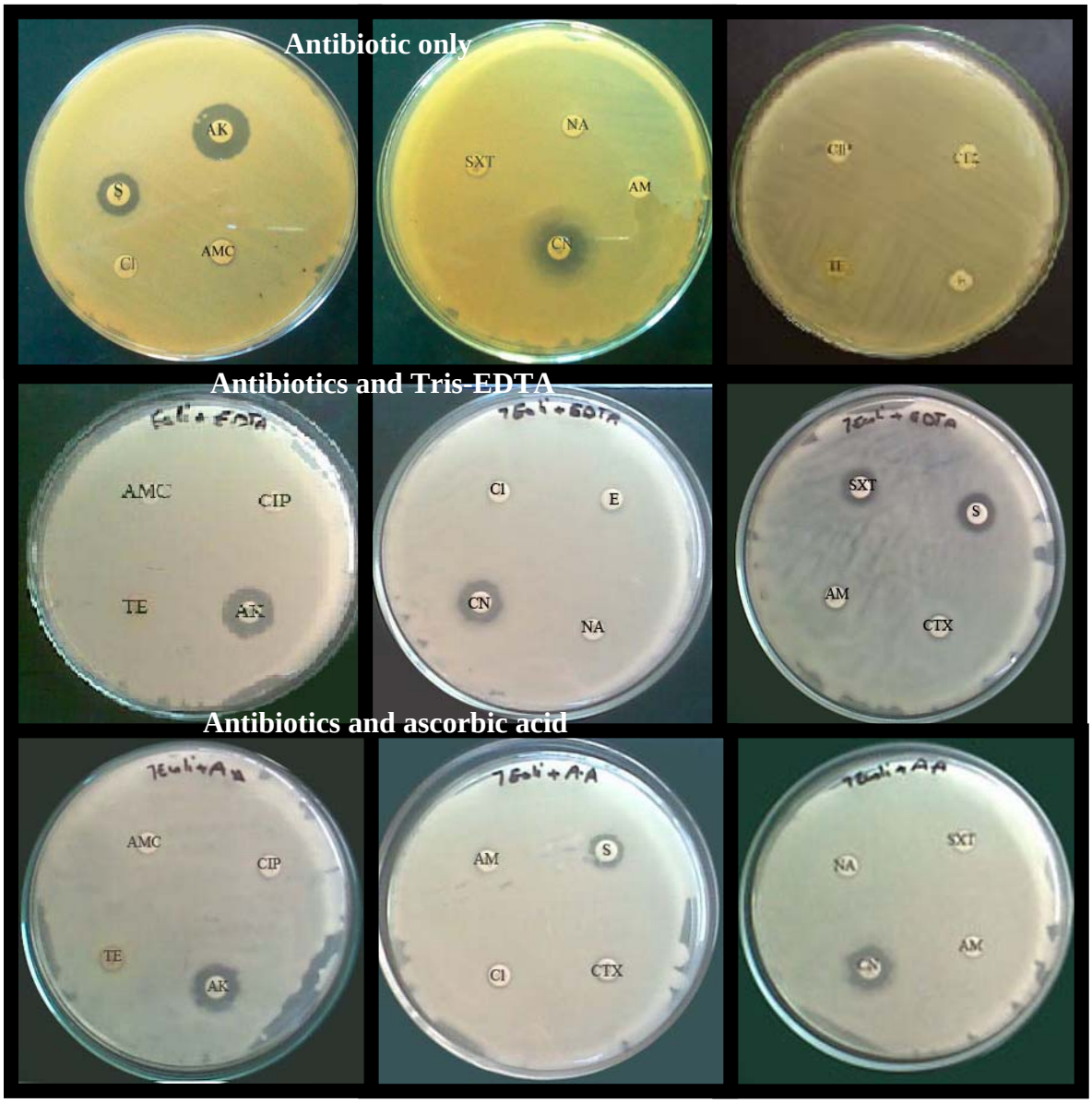
Table (3-14). Effect of combination of ascorbic acid with antibiotics against bacterial isolates using disk diffusion assay.

Isolate	Antibiotic	Inhibition zone diameter (mm)	
		Antibiotics alone	Antibiotics and ascorbate
Ps 1	CIP	13	10
	S	10	13
	AK	14	15
	CTX	0	12
	CN	9	11
Ps 6	CIP	27	17
	AK	0	10
Ps 15	CIP	23	14
	AK	17	19
	CTX	0	14
	AM	0	11
	CN	0	10
Ps 19	CL	0	14
	CIP	27	15
	S	0	14
	CTX	0	15
	E	0	18
	AM	0	12
	SXT	0	12
	CN	0	11
	NA	0	13

Pr 28	CIP	40	0
	AMC	0	13
	CTX	20	0
	TE	0	18
	CN	15	13
	NA	20	0
E. coli 30	CN	10	13
Staph. aurous 38	CL	0	28
	CIP	20	26
	S	10	15
	AMC	0	22
	AK	12	21
	CTX	0	29
	TE	18	36
	E	14	21
	AM	0	20
	SXT	19	28
	CN	12	22
	NA	0	13
Staph. epidermidis 43	CIP	0	21
	AMC	0	12
	CTX	0	18
	TE	0	14
	AM	0	12
	SXT	0	9
	CN	0	14
	NA	0	17
Staph. epidermidis 51	CIP	22	29
	AMC	20	23
	AK	17	22
	CTX	0	18
	TE	0	15

	AM	0	14
	SXT	18	20
	CN	0	18
	NA	0	16

***Ps*: *Pseudomonas aeruginosa*, *Pr*: *Proteus mirabilis*, *Staph. aureus*:** *Staphylococcus aureus*, ***Staph. epidermidis*:** *Staphylococcus epidermidis* ***E. coli*:** *Escherichia coli.*



(A)

Figure (3-3) Effect of antibiotics alone and in combination with EDTA (0.006-0.0018 mg/ml) or ascorbic acid (10mg/ml) against different type of bacteria

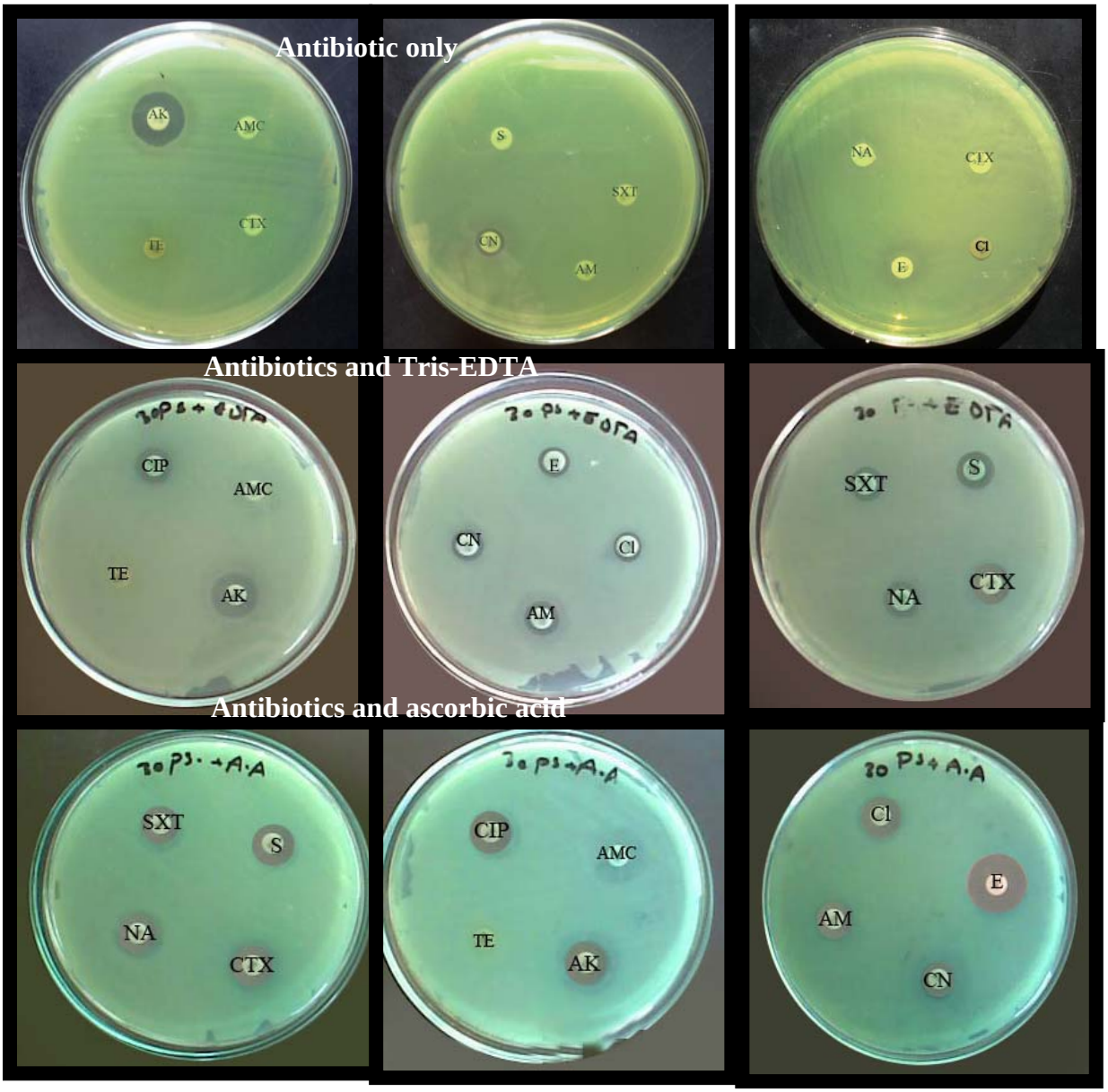
A: ***E.coli.***

B: ***Pseudomonas aeruginosa.***

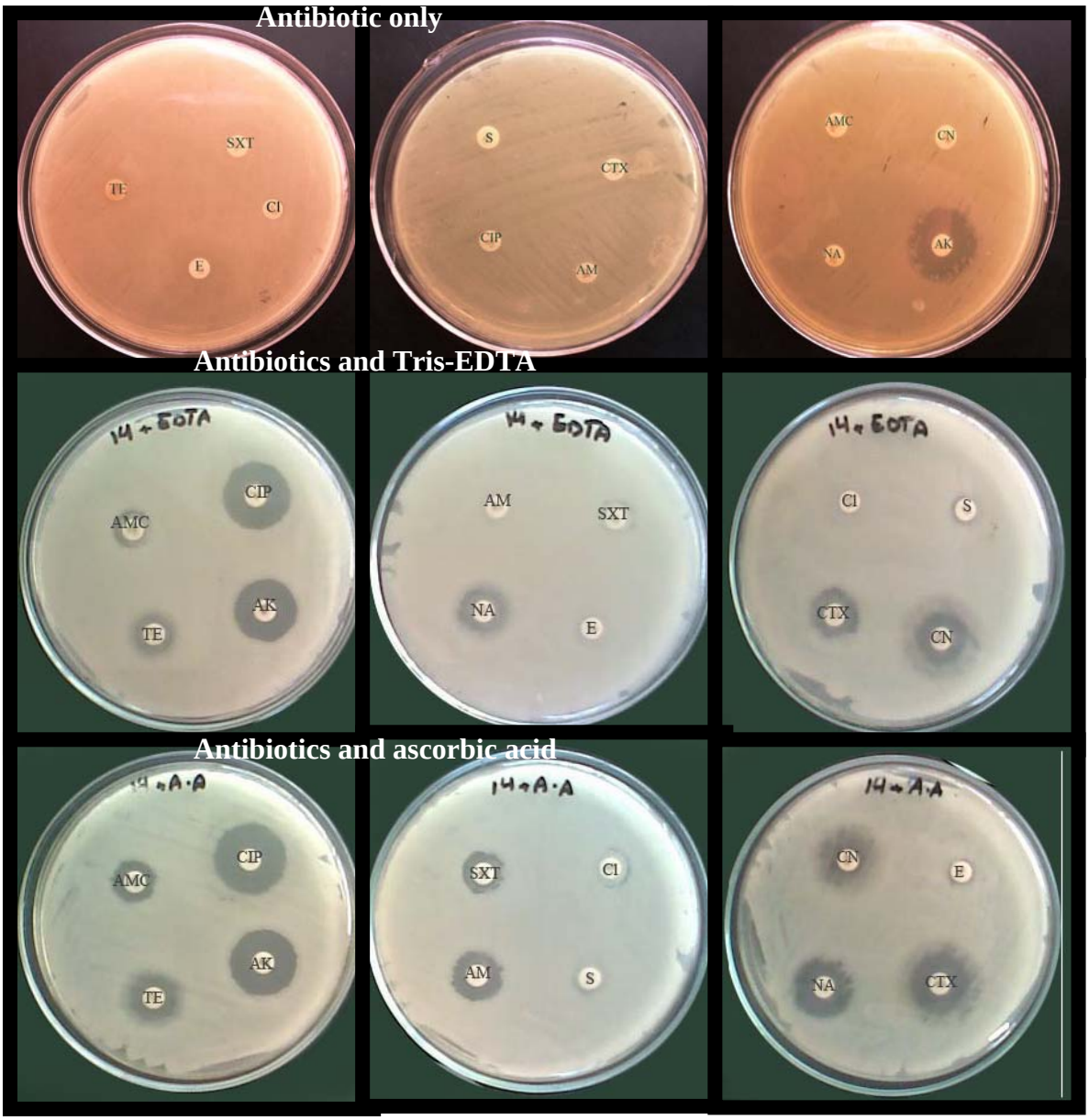
C: ***Staph. epedemidis.***

D: ***Proteus maribilius.***

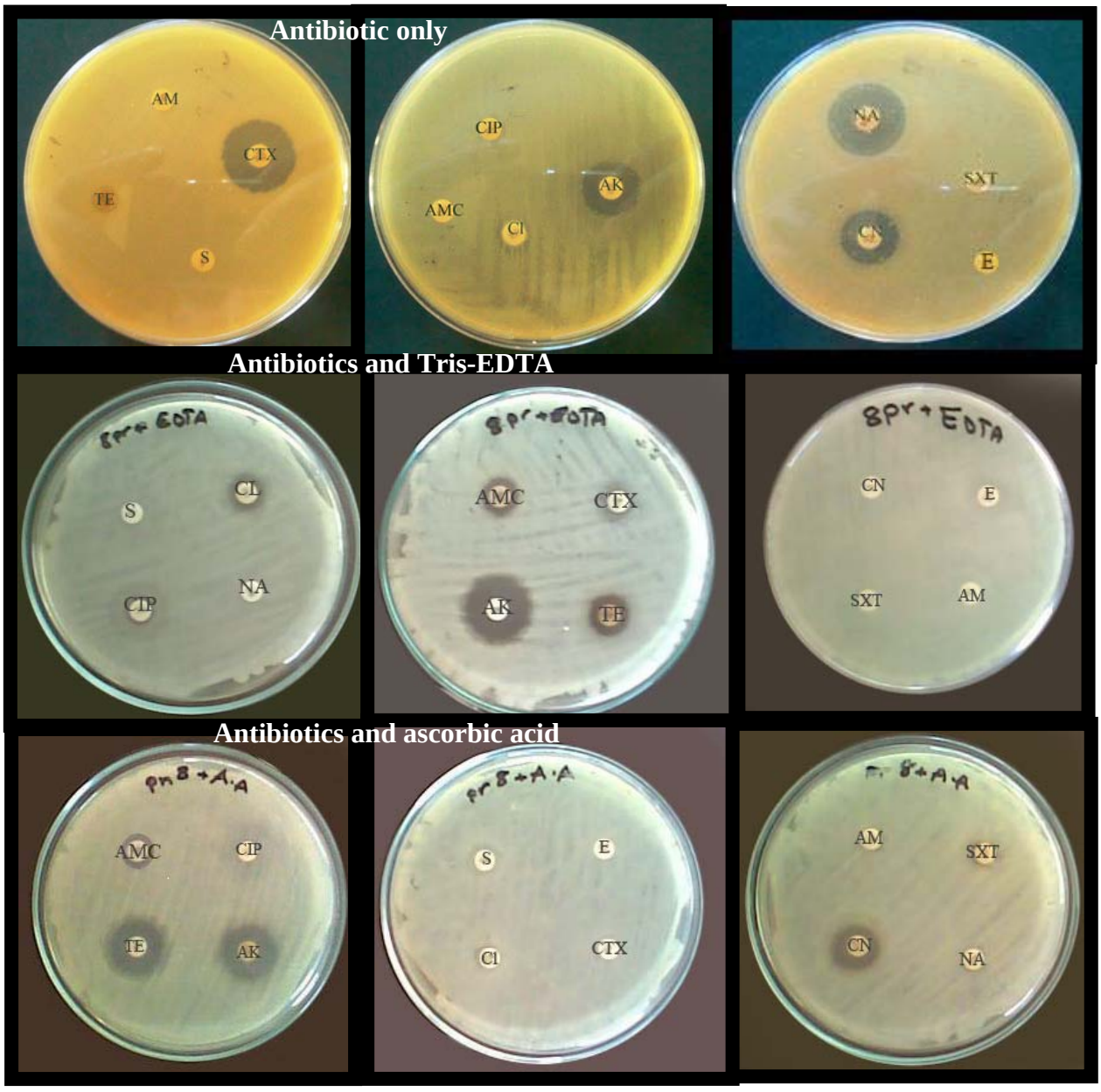
E: ***Staph aureus.***


Antibiotic only
AK
AMC
TE
CTX
S
SXT
CN
AM
NA
CTX
E
Cl
Antibiotics and Tris-EDTA
CIP
AMC
TE
AK
E
CN
Cl
AM
SXT
S
NA
CTX
Antibiotics and ascorbic acid
SXT
S
NA
CTX
CIP
AMC
TE
AK
Cl
E
AM
CN

(B)


Antibiotic only
SXT
TE
CI
E
S
CTX
CIP
AM
AMC
CN
NA
AK
Antibiotics and Tris-EDTA
14 + EDTA
CIP
AMC
AK
TE
14 + EDTA
AM
SXT
NA
E
14 + EDTA
CI
S
CTX
CN
Antibiotics and ascorbic acid
14 + A·A
CIP
AMC
AK
TE
14 + A·A
SXT
CI
AM
S
14 + A·A
CN
E
NA
CTX

(C)


Antibiotic only
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CTX
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CIP
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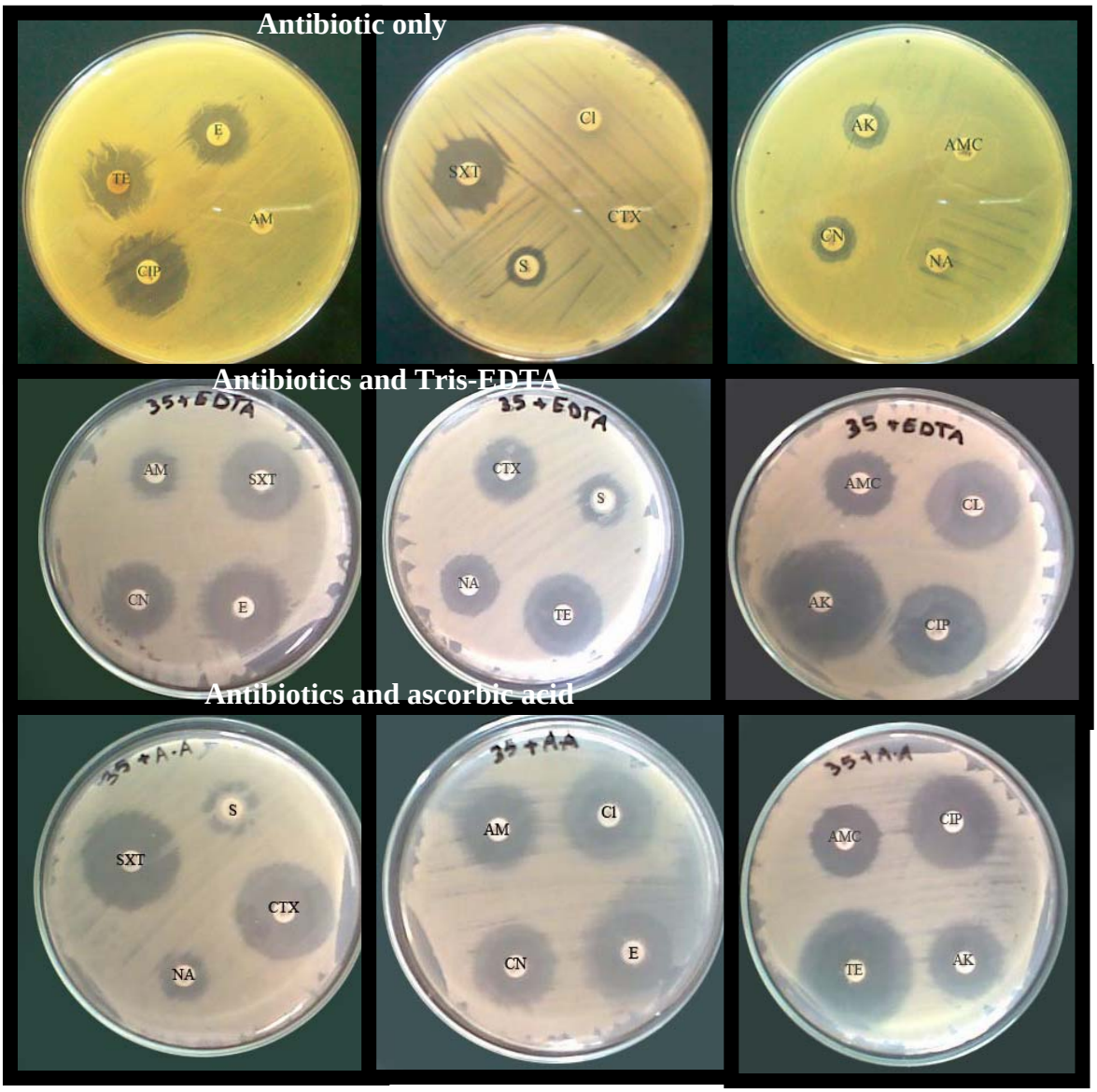
SXT
CN
E
Antibiotics and Tris-EDTA
CI
S
CIP
NA
AMC
CTX
AK
TE
CN
E
SXT
AM
Antibiotics and ascorbic acid
AMC
CIP
TE
AK
S
E
CI
CTX
AM
SXT
CN
NA

(D)


Antibiotic only
E
TE
AM
CIP
CI
SXT
CTX
S
AK
AMC
CN
NA
Antibiotics and Tris-EDTA
35+EDTA
AM
SXT
CN
E
35+EDTA
CTX
S
NA
TE
35+EDTA
AMC
CL
AK
CIP
Antibiotics and ascorbic acid
35+A·A
S
SXT
CTX
NA
35+A·A
AM
CI
CN
E
35+A·A
AMC
CIP
TE
AK

(E)

Chapter Four

Conclusions And Recommendations

Conclusions

1. *Pseudomonas aeuginosea* was the most common tested bacteria that causing otitis media infection.
2. Ciprofloxacin was the most effective antibiotic against bacterial isolates from otitis media infection followed by amikacin, then gentamycin.
3. Tris-EDTA and Ascorbic acid showed synergistic effect with antibiotics against gram positive bacteria, causing otitis media.
4. Tris-EDTA and Ascorbic acid showed antagonistic effect when used with aminoglycosides and quinolone antibiotics against gram negative bacteria.

Recommendations

1. Studying the synergistic effects between Tris-EDTA and ascorbic acid individually with antibiotics against other microorganisms such as: anaerobic bacteria, viruses and fungi.
2. Further studies are required for possible use of Tris-EDTA with antibiotics in drop with intact tympanic membrane in patients with otitis media.
3. Further studies are required for possible use of ascorbic acid at different pH with antibiotics in patients with otitis media.

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تاثير Tris-EDTA و فيتامين C على زيادة فعالية المضادات الحيوية ضد البكتريا المعزولة من التهاب الاذن الوسطى

رسالة

مقدمة إلى كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

اروى حمودي كريم

بكالوريوس تقانة احيائية- جامعة النهرين (٢٠٠٥)

ذو القعدة
١٤٢٩

تشرين الثاني
٢٠٠٨

الخلاصة

- أجريت هذه الدراسة لتسليط الضوء على بعض المواد المستعملة لزيادة فعالية المضادات الحيوية ضد البكتريا المعزولة من المرضى المصابين بالتهاب الاذن الوسطى.
- جمعت لهذا الغرض ٨٤ عينة سريريه من مرضى مصابين بالتهاب الاذن الوسطى بمختلف الاعمار ولكلا الجنسين في مستشفى الكاظمية التعليمي ومختبر الصحة المركزي من الفترة كانون الاول ٢٠٠٦- ايار ٢٠٠٧.
- تمكنت ٥٩ عينةعلى النمو،حيث شخصت ٢٥ منها *Pseudomonas areuginosa* ١٥، *Staphylococcus epidermidis* ٦، *Staphylococcus aureus* ٥، *Escherichia coli* ٣، *Proteus maribilis* و ٥ من الفطريات. *Pseudomonas areuginosa* تعتبر من الممرضات الرئيسية لالتهاب الاذن الوسطى.

المرضى الاكثر اصابة بمرض الاذن الوسطى هم بين الاعمار (١– ١٠) سنة. الذكور أكثر إصابة من الاناث وإصابة الإذن اليمنى أكثر من اليسرى. انتشار الاصابة في فصل الشتاء اكثر من أي فصل اخر. اصابة الاذن بنوع واحد من البكتريا اكثر من الاصابة بنوعين او ا كثر.

- اختبرت حساسية هذه العزلات لمضادات حياتية مختلفة باستخدام طريقة الاقراص وقد اظهرت النتائج بان المضاد Ciprofloxacin هو الاكثر فعالية ثم يعقبه على التوالي amikacin و gentamycin.
- اخضعت (٩) عزلات (اعتمادا على تعدد مقاومتها للمضادات الحياتية) لتحديد التراكيز المثبطة الدنيا MIC للمضادات Streptomycin, Ciprofloxacin, Cephalexin, Amikacin, Cefotaxim, Tetracyclin, Erythromycin, Ampicillin, Gentamycin, Nalidixic acid. حيث اظهرت النتائج بان المضاد Ciprofloxacin ادى الى تثبيط نموها بادنى التراكيز ا مقارنة بالمضادات الاخرى.

- تمت دراسة تأثير مزج المضادات الحياتية مع Tris-EDTA و Ascorbic acid ضد عزلات البكتريا التسع المختلفة باستخدام طريقة أقراص المضادات الحياتية بعد مزج Tris-EDTA مرة و Ascorbic acid مرة أخرى مع المضاد الحيوي و أظهرت النتائج بانه كان للبكتريا السالبة والموجبة تأثيرا تأزريا بين كل من Tris-EDTA و Ascorbic acid مع tetracycline, aminoglycosides, SXT, quinolone, erythromycin, β-lactam، فيما اعطت بعض العزلات تأثيرا عكسيا لكل من Tris-EDTA و Ascorbic acidمع aminoglycosides, quinolone.