PLASMID PROFILE OF BACTERIA ISOLATES FROM PATIENTS WITH EAR INFECTION AND ASSOCIATED PATIENT DEMOGRAPHIC FACTORS AT A TERTIARY HEALTH CARE FACILITY

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY AND BIOTECHNOLOGY, FACULTY OF PHARMACY, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL FULFILMENT OF THE REQIREMENTS FOR THE AWARD OF THE DOCTOR OF PHARMACY (PHARM.D) DEGREE

APRIL, 2024

CERTIFICATION

This is to certify that this work was carried out by DOTUN ANDREW AWOLEYE in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin city, Nigeria

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CERTIFICATION OF THESIS ON PLAGIARISM

We the undersigned attest and declare that the thesis of DOTUN ANDREW AWOLEYE Titled: **PLASMID PROFILE OF BACTERIA ISOLATES O FROM PATIENTS WITH EAR INFECTION AND ASSOCIATED PATIENT DEMOGRAPHIC FACTORS AT A TERTIARY HEALTH CARE FACILITY.** Has successfully passed the anti-plagiarism test and does not violate any copy right regulations.

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DEDICATION

To my beloved mum Mrs Awoleye F.O, I deeply appreciate you, for giving me the enablement and providing the resources to undertake this important journey and also for being as a source of encouragement through it all. I Also dedicate this Research project to God Almighty who has indeed kept and guided me all through this journey.

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ABSTRACT

Background: Ear infections, also known as otitis media, are a common type of bacterial infection that mostly affect children. Plasmid profiling is a molecular technique used to identify and characterize the types of plasmids carried by bacterial isolates. This technique has a wide range of applications in microbiology, including the study of bacterial epidemiology, antibiotic resistance, and gene transfer. It can provide valuable information about the genetic diversity of bacterial populations and the mechanisms by which bacteria acquire and transfer resistance genes.

Methods: The study evaluated a hundred and twenty seven patients from the Ear, Nose and Throat department at the University of Benin Teaching Hospital, Benin city. Patient's data collected were age, gender, alcohol history, smoking history, medical history, Frequency of symptoms, ears affected, use of cotton bud, ear cleaning frequency among others. Isolates obtained were subjected to antimicrobial susceptibility testing and plasmid curing with 0.1mL and 0.4mL Ethidium bromide as the curing agent using standard agar disc diffusion method.

Results: A total of 73.68% of our study participants were female, and 14.47% were male. Participants aged 21-25 had the highest occurrence (34.6%), linked to the patients ear cleaning habits. The prevalence of *Staphylococcus* spp and *Pseudomonas aeruginosa* from both aerobic and anaerobic Isolate was the highest (49.33% and 47.06%) in the bacterial isolates obtained from study participants after incubation.The most prevalent mult-idrug resistant isolate was *Staphylococcus aureus* (25%) for aerobic isolate and Pseudomonas spp for anaerobic Isolate. Fluoroqinolones was the most effective antibiotics used for antimicrobial susceptibility testing. Post curing with 0.1mg/ml and 0.4mg/ml Ethidium bromide,33.33%,100% and 0% was cured after further antimicrobial susceptibility testing with Fluoroqinolones, Macrolides and Penicillin) respectively.

Conclusion:The presence of plasmid-mediated multidrug resistance genes in bacteria isolates from patients with ear infection underscores the need for antibiograms and responsible antibiotic usage in the management of ear infection.

CHAPTER ONE

1.0 INTRODUCTION

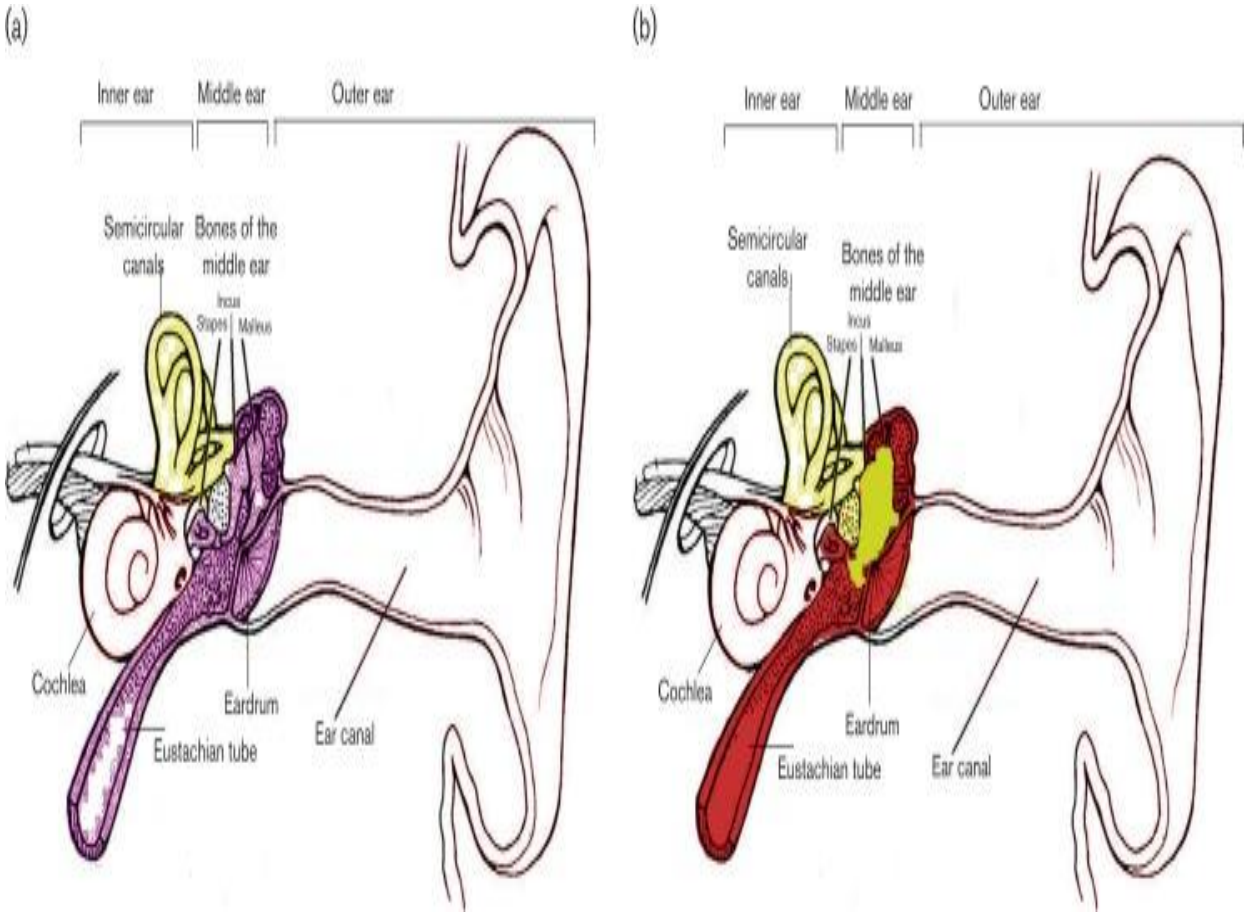
1.1 Background of the study

Ear is the organ of hearing and balance which has three parts, such as outer, middle, and inner ear. The outer ear collects sound waves, which move through the ear canal to the tympanic membrane, commonly called the eardrum. The inner ear is filled with fluid, here hair like structures stimulate nerves to change sound waves into electrochemical impulses (Guyton, 1999). The mastoid process of the temporal bone houses the middle ear cavity. The tympanic membrane and the inner ear are separated by the cavity.The Eustachian tube allows the nasopharynx to extend into the middle ear cavity. (Masters *et al.,* 1960). Otitis externa, otitis media, and otitis internal are three different types of ear infections that can cause significant public health concern in developing countries due to their high disease burden and negative impacts on patients, families, and the healthcare system.

Otitis externa is the first kind, which affects the ear canal and outer ear. In external otitis, the ear is painful when touched and it's also called swimmers ear. Otitis media is the second type in which the ear is contaminated with fluid behind the ear drum, in the habitually air-filled space of middle ear. The infection of middle ear is very common in childhood, sometimes requires a surgical procedure called myringotomy and tube insertion. Eosinophilic otitis media is a newly recognized entity causing intractable middle ear pathology. This condition is characterized by excessive accumulation of eosinophils in the middle ear cavity and is associated with persistent middle ear effusion. These patients usually suffer from bronchial asthma. These secretions are highly viscous and the middle ear mucosa appears pinkish in colour (Lino *et al*., 2005). Otitis internal, which affects the sensory organs in the inner ear, is the third one. Vertigo, also known as labyrinthitis, is a typical sign of inner ear inflammation.

(Sabella et al, 2005). A complication of major ear infections such as Otitis media varies depending on the duration of microbial colonization, severity of infection and associated microorganisms. Depending on the clinical presentation, otitis media can be subdivided into 2 types such as chronic suppurative otitis media (CSOM) and chronic otitis media with cholesteatoma (AMO) (Oni *et al*., 2002). in suppurative chronic otitis media, there is a hole in the eardrum and an infection in the middle ear. cloudy and sometimes foul- smelling fluid drains out through the opening(Sharma et al,2004). in chronic otitis media with cholesteatoma, there is a growth(tumor) in the middle ear composed of skin cells and debris that can occasionally result from a chronic hole in the eardrum. when the eustachian tube is obstructed but there is no hole,cholesteatoma can still develop. Ear leakage and hearing loss are two possible outcomes of cholesteatoma. The mastoid bone behind the middle ear and the middle ear structures will erode due to the size of the cholesteatomas. Another type such as otitis media with effusion(OME) is also common .

Fig 1.0



- American Academy of Otolaryngology—Head and Neck Surgery Foundation (AAO-HNSF). Otitis Media. 2015.

Schematic representation of the ear under normal and CSOM conditions. (a) Under normal conditions, the middle ear cavity is clear and empty.(b) In contrast, the middle ear becomes red and inflamed with the presence of fluid under CSOM conditions. The red colour denotes inflammation, while yellow indicates fluid during CSOM .

1.2 Pathophysiology of ear infection

It is possible for fungus, viruses, and bacteria to cause ear infections. According to Bluestone and Klein (1990), bacteria that are present in the skin of the external ear and can penetrate the middle ear through a chronic perforation are the main cause of ear infections. Bilateral otomycosis has been linked to the opportunistic filamentous fungus Aspergillus niger (Gugnai et al,1989). Deep within the external canal, Aspergillus niger thrives on cerumen, epithelium scales, and debris. Pruritis, or irritation of the external ear's surface layer, is a risk factor for bacterial colonization. The accumulation of these inflammatory materials, cerumen, and fungal debris causes plug formation, which is highly significant and typically results in reduced hearing ability. Membrane erosion may occur on the surface. Treatment for an ear infection with antibiotics is necessary to prevent further infection or hearing. On the other hand, the usage of antibiotic ear drops may have suppressed the bacterial flora, allowing the fungal flora to develop. This might account for the presence of fungus. Fungal superinfection is likely to become more common as a result, and even less virulent fungi may become more opportunistic (Koopman et al., 2008). Physicians must, however, take into account the vast range of pathogens, including bacteria, viruses, and fungi, when treating patients with ear infections, and then tailor their treatment to the specific pathogen causing the illness. Severe sickness or drug resistance may result from improper antibiotic treatment (Tanno et al., 1999).

Many factors like Eustachian tube dysfunction and susceptibility to upper respiratory tract infection may contribute to pathogenesis of otitis media (Ahmad *et al*., 1999). Otitis media is an inflammatory condition of the middle ear that is initiated by functional or mechanical obstruction of the Eustachian tube. It leads to alteration in the partial pressure of middle ear gases, resulting in negative middle ear pressure. Nasopharyngeal bacteria may invade the middle ear space via the obstructed Eustachian tube and replicate within the serous middle ear fluid. Bacterial and inflammatory host cell products are released into middle ear effusion

and tissues, attracting peripheral blood leukocytes and leading to a cascade of acute inflammatory events that result in symptomatic acute otitis media (Kathleen *et al*., 1999). The most common cause of eustachian tube dysfunction is a viral process. Early infancy is a more complex phase for this dysfunction because of the tube's horizontal structure, which increases the impact of middle ear fluid flow . Infections in the respiratory system accompany middle ear effusion (MEE) in the pathophysiology of ear infections. While the fluid collection under the Eustachian tube and tympanic membrane (TM) is sterile, the secretions coming from the nasopharynx are not. Microbial reflux into the middle ear region occurs when the Eustachian tube briefly relaxes. This might result in colonization and bacterial adherence, which could then cause the inflammatory process associated with AOM. Abraham and Labbok(2011).

1.3 Symptoms of ear infection

Most often, an ear infection takes two to seven days to manifest symptoms. In addition to the primary symptom of ear discomfort, other symptoms include fever, vomiting, ear fluid leakage, fussiness, clumsiness, and ringing in the ears (Morris, 2004

1.4 Epidemiology of ear infection

Less than 20% of individuals with acute otitis media appear as adults. In addition to fever and appetite loss, AOM causes ear discomfort in young children, which can cause straining at the ear, increased screaming, and restless nights. (Del-Mar *et al*., 1997). It has long been known that a greater prevalence and severity of recurrent AOM are linked to poverty level. Crowding, poor cleanliness, nutritional condition, restricted access to medical treatment, and pharmaceutical availability are potential contributing factors(Vakharia et al, 2010).

Although Acute otitis media can occur at any age, it primarily affects the young, with cases most frequently occurring in three months to three year age range. At least 60% of children have had at least one episode of AOM by the time they are a year old, and 17% have had at

least three episodes (Teele et al,1989). There is enough evidence to conclude that respiratory viruses play a significant role in the etiology and pathophysiology of acute ear infections, even though these infections are often diagnosed as bacterial infections (Ruuskane et al., 1991). Different geographic locations have distinct ear infection etiologies and prevalence rates (Brook and frazier, 1996). Based on a survey conducted by the World Health Organization (WHO), nations can be classified as low- or high-ear infection-prevalence when the rate of ear infections in children falls between 1% and 6%.. Between 50% and 85% of children are estimated to have had acute otitis media at least once by the time they are three years old, with the peak incidence occurring between six and fifteen months of age (Klein, 2015). In 2015, the World Health Organization estimated that 328 million people and 32 million children worldwide suffered from a deafening hearing loss. The areas of South Asia, Asia-Pacific, and Sub-Saharan Africa have the greatest frequency. While many cases of hearing loss may be cured, half of all cases can be prevented by primary prevention. Untreated ear infections, sometimes accompanied by drainage from the ear, are a major cause of hearing loss in younger people, especially in low- and middle-income countries. Hearing loss can also result from infectious disorders that can be prevented by vaccination, such as meningitis, rubella, measles, or mumps (Fauci et al., 2008).

1.4 Diagnosis of ear infection

There are numerous methods, including various tests, for diagnosing an ear infection. According to Haiharan et al. (1995), the most popular preferential test is the pneumatic otoscope, which involves passing air into the ear to view the movement of the ear drum. Other diagnostic instruments include blood tests, tympanometry, and hearing tests. Only in cases where immunodeficiency is a primary cause of ear infection is a blood test for diagnosis recommended. A few defensive measures, such as vaccination, appropriate child

care, quitting smoking, and maintaining hygienic conditions, could be implemented to prevent ear infections (Tagg and Dierksen, 2003).

1.5 Management of ear infection

Acute otitis media is generally a self-limiting infection in otherwise healthy children. In 90% of these children, ear pain fully resolves within seven to eight days (Thompson et al,2017) and in 80% of children, the worst symptoms settle within three days without antibiotics(Venekamp et al,2015). Symptomatic treatment with analgesics and watchful waiting form the mainstay of management in otherwise healthy children with acute otitis media presenting with mild ear pain and who are systemically well. You might offer a back-up (delayed) antibiotic prescription, to be used if symptoms do not improve within three days or worsen substantially at any time. Patients with mild symptoms of otitis externa may benefit from a reasonable treatment approach that involves using a topical otic preparation without culture. Given its efficacy and affordability, an otic preparation comprising neomycin, polymyxin B, and hydrocortisone would be the first choice if the tympanic membrane is unharmed and there is no risk of aminoglycoside hypersensitivity. If the tympanic membrane is not intact or its condition cannot be visually assessed, ofloxacin and ciprofloxacin/dexamethasone (Ciprodex), which are approved for use in the middle ear, should be used (Rosenfeld et al., 2006). Methicillin-resistant Staphylococcus aureus (MRSA) and penicillin-resistant Streptococcus pneumoniae are two pathogens that make treating serious infections caused by Gram-positive bacteria more challenging (Menichetti, 2005). Immunocompromised patients may develop extremely serious illness from *Pseudomonas aeruginosa* and if not appropriately treated in a timely manner, could become life-threatening. Cephalosporins are extremely sensitive to both gram positive and gram negative bacteria. The most effective treatment for ear infections is combination therapy, which can be used to treat them effectively and quickly with fewer chances of resistance building up. It could also be

treated with a single antibiotic, such as cephalosporins; however, infections brought on by Pseudomonas species require a combination of antibiotics to be effectively treated (Tano et al., 1999)Keeping an ear infection at low levels .The use of regular doses of paracetamol or ibuprofen at an age-appropriate dose for the management of pain is recommended(Vanuum et al,2019).

1.6 Prevention of ear infection

In order to reduce the effects of this illness, the need for effective antibiotics prescription are crucial. However, the emergence of bacterial resistance to commonly prescribed antibiotics has limited the therapeutic use of antibiotics in the treatment of ear infection. Promising candidate antigens for vaccination have been found in M. catarrhalis, nontypeable H. influenza, and S. pneumoniae, the most common cause of ear infections. Targeting the most common cause of ear infections, the pneumococcal conjugate vaccine has shown promise in treating invasive pneumococcal disease, or pneumonia (Pelton et al., 2013).

1.7 Global burden of ear infection

The World Health Organization's World Report on Hearing warns that by 2050, nearly 2.5 billion people will have some degree of hearing loss worldwide, and at least 700 million of these people will need access to hearing care and other rehabilitation services if no action is taken (WHO,2021). Otitis media is one of the major causes of hearing loss and deafness, especially in children under the age of five and in individuals in developing countries .Global Burden of Disease (GBD) Collaboration released hearing loss data in 2019, which included otitis media. However, the long-term trends of incidence and disability-adjusted life years (DALYs) associated with otitis media, particularly in low- and middle-income countries, remain unclear). Trend analysis could uncover insights regarding the impact on policies or

interventions, providing specific recommendations for healthcare providers, policymakers, and researchers.

Hearing loss has an enormous global economic impact, estimated at 750 billion international dollars annually, with 63%–73% of these costs incurred in low-income or middle-income countries (LMICs)(WHO,2017). Hearing impairment and disease-related hearing loss can have significant consequences for the individual, and can be a substantial financial drain to society. The presence and impact of disabling hearing loss is unequally distributed across the world, with the burden of hearing loss (both individual and societal) being greatest in low-resource countries, where access to education, medications, technology and interventions is limited. Given the social, emotional and significant economic impact of hearing loss, it is important that resources are targeted towards minimising the load. Costs include those related to healthcare, educational support, loss of productivity, and social costs associated with the stigma of having hearing loss. Several studies report that in low- and middle-income countries, 50 per cent of otitis media cases will also have an associated hearing impairment, although some studies have reported estimates as high as 60–100 percent(Bastos et al,1995). The degree of hearing loss associated with otitis media is commonly mild(Robert et al,2004). Depending on the degree of hearing loss and the fluctuating nature of the middle-ear fluid, speech sounds can be distorted and speech intelligibility may be compromised(Clarkson et al,1989), which can significantly delay a child's ability to acquire speech and language. In addition, higher-level auditory processing, such as localisation and listening ability in the presence of background noise, may be affected, which can have a detrimental impact on a child's learning and education. Consequently, a data-driven analysis characterizing global otitis media disease burden in relation to national socioeconomic status provides an important perspective for physicians, patients, and policymakers.

1.8 Antimicrobial resistance trend

Widespread antibiotic use has promoted the emergence of antibiotic resistant pathogens, including multidrug resistant strains. Resistance is spreading rapidly, particularly in hospitals, where various bacteria can come in close contact with one another, spreading the resistance traits in the process. Since bacteria share resistance genes, nosocomial antibiotic resistance can spread to surrounding communities. At present, the understanding of various drivers of antibiotic

resistance is the key to addressing this global concern. The occurrence of resistance in microbes is a natural process and additionally important factors which are potent drivers of antibiotic resistance include sanitation settings ,infection control standards, water hygiene systems, drug quality, diagnostics and therapeutics, and travel or migration quarantine. In addition to the mutation in various genes residing on the chromosome of the microorganism, exchange of genetic material between organisms plays a vital role in the distribution of antibiotic resistance(Holmes et al,2016).The various antibiotic resistance mechanism include alteration/modification of the target site, degradation of the antibiotic molecule and reduction of effective intracellular antibiotic concentration as a result of decreased permeability and energy- dependent(or active) efflux. Resistance genes are either carried on the chromosomes of wild-type bacteria or on elements of extrachromosomal ,sometimes extraneous origins, such as resistance plasmids and transposons. The effective use of an antimicrobial agent is undermined due to the possible tolerance or resistance developed from the very initial time this compound is used. This is true for antimicrobial agents used to treat bacterial, viral, fungal, and parasitic infections. Several physiological and biochemical mechanisms may steer this developing resistance. The intricacy of all the mechanisms associated with the emergence and distribution of the resistance should not be overplayed. Numerous important organizations, like the Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America, World Economic Forum, and the World Health Organization

(WHO) have declared antibiotic resistance to be a “global public health concern. antibiotic resistance shows no signs of decline ,though it may perhaps shift direction. The etiology of antibiotic resistance is multifaceted, and its consequences pose an impact across the globe. Numerous attempts have been made to delineate the diverse aspects of antibiotic resistance and possible solutions required to deal with this global challenge have been tried.

Bacteria resistance to antibiotic may be defined as the inability of an antibiotic to inhibit bacteria growth effectively .Microorganisms generally acquire antibiotics resistance by genetic changes, but sometimes they do so by non genetic mechanisms(Munita and Aries,2016). At present, the multifaceted etiology of antibiotic resistance has many factors which are at play. These include inadequate regulations and usage imprecisions, awareness deficiency in best practices which steers undue or inept use of antibiotics, use of antibiotics as a poultry and livestock growth promoter rather than to control infection, and online marketing which made the unrestricted availability of low-grade antibiotics very accessible(spellberg et al,2014). Additionally, indistinguishable resistant mechanisms have been found in bacteria isolated from humans or animals. Resistant bacteria and mobile genetic elements (MGEs) may make their way from animals to humans through various means(Molbak,2004) .

1.9 Superbugs and super-resistance

The word “superbugs” denotes microbes with higher morbidity and mortality rate increased due to several mutations providing resistance to various classes of antibiotics. Therapeutic preferences for these resistant microbes are less, and these are associated with a prolonged stay at hospital and increased economic cost. In certain cases, super-resistant strains have developed increased virulence and improved transmissibility as well(Davies and Davies,2010). Due to aberrant use of antibiotics, numerous bacterial human pathogens have evolved into MDR types. *S. aureus* is considered as the most notorious superbug. It is a nasal

commensal of humans and can cause common skin infections. At present, CA-MRSA with increased acquired virulence has emerged as a major community concern.

1.9.1Antibiotic resistance and virulence

Human skin, mucous membranes, and the interior of the body are all susceptible to bacteria. Bacteria comprise a diverse range of species, many of which are beneficial commensals and a select few of which are absolutely necessary. But some are recognized pathogens that infect people; they are capable of colonizing, invading, and damaging the host tissue (Beceiro et al., 2013). A pathogen possesses several characteristics that enable it to become more harmful, a quality known as virulence. Pathogenicity is the capacity of a bacteria to cause disease. The two most crucial characteristics that aid a pathogen in causing a disease are toxicity and invasiveness employed at this time to the host's immune system (Martinez et al, 2002).The host's immune system and pathogenicity may have an impact on the final balance of a bacterial disease's progression. It's possible that the coevolution of the bacteria and host extends beyond millions of years. During this time, pathogens have modified their virulence to adapt to the host's immune system (Martinez et al, 2002). The evolution of antibiotic resistance, which has primarily occurred in the last five decades following the discovery of antibiotics, stands in contrast to this. According to Fair et al. (2014), resistance and virulence are thought to have changed over different historical periods . Despite their diverse evolutionary histories, these mechanisms have much in common, and both are essential to the survival of bacteria in adverse settings. Both virulence and resistance factors transfer determinants between genera or species through a process known as horizontal gene transfer. The transfer of MGEs may be the primary genetic process for the distribution and co-selection of resistance and virulence genes, even though other processes like adaptive or compensatory mutations may also be important (Handel et al., 2008).

1.9.2 Types of resistance

Natural resistance

Two types of natural resistance exist in bacteria: intrinsic resistance, which is expressed equally across the species, and induced resistance, which is expressed to higher levels only after the bacteria are exposed to an antibiotic. A characteristic that is common to all members of a bacterial species, unrelated to horizontal gene transfer, and not influenced by prior antibiotic exposure is known as intrinsic resistance (Cox and Wright, 2013). Reduced permeability of the outer membrane (most especially, the lipopolysaccharide, or LPS, in gram negative bacteria) and the inherent functioning of efflux pumps are the most prevalent bacterial mechanisms causing intrinsic resistance. According to Fajardo et al. (2008), multidrug-efflux pumps are another typical mechanism of induced resistance.

Acquired resistance

All of the primary methods by which bacteria acquire genetic material—transposition, conjugation, and transformation—collectively known as horizontal gene transfer, or HGT—can result in the acquisition of genetic material that confers resistance. In addition, the bacteria may undergo mutations to its own chromosomal DNA. The acquisition could be long-term or short-term. The most frequent method of acquiring foreign genetic material is by plasmid-mediated transmission of resistance genes; bacteriophage-borne transmission is somewhat rare.

Since they are naturally competent, certain bacteria, like Acinetobacter spp., can take up genetic material straight from their surroundings. Internal insertion sequences and integrins have the ability to rearrange genetic material, and common sources of genetic changes in bacteria include hunger, UV radiation, chemicals, and other stresses. (substitutions, deletions etc.). One mutation occurs in bacteria on average every 106 to 109 cell divisions, and the majority of these mutations are harmful to the cell [Coculescu, 2009]. Only a few different

categories of genes—drug targets, drug transporters, regulators that regulate drug transporters, and antibiotic-modifying enzymes—typically have mutations that contribute to antibiotic resistance.

Furthermore, the organism bears a cost for several modifications that contribute to antibiotic resistance. The fact that using these medications causes resistance to grow is one major mystery surrounding antimicrobial resistance. In addition to selecting for bacteria that are hyper-mutatable strains (increasing the mutation rate), using low or very low concentrations of antimicrobials (sub-inhibitory) can also increase the ability of bacteria to acquire resistance to other antimicrobial agents and encourage the movement of mobile genetic elements [Blazquez et al., 2015].

1.9.3 Different Broadways of acquiring resistance

The role of mobile genetic element

MGEs mediate the transfer of chromosomal DNA or they themselves move between cells to induce DNA transfer between bacteria. Certain HGT methods, such as natural transformation (Johnson et al., 2014), are independent of MGEs (Garcia et al., 2017). However, it is unclear how important these mechanisms are for the acquisition of novel genes in bacteria as a whole. Depending on their autonomous horizontal (conjugation or viral particles) or vertical (extrachromosomal or integrative) transmission modes, MGEs can be categorized. Each form of MGE has a significant amount of genetic mutation, which can make it difficult to characterize and identify them. Moreover, some MGEs create intricate ecological dynamics within populations by parasitizing or competing with other MGEs. The key drivers in horizontal gene transfer (HGT) are mobile genetic elements (MGEs), which include plasmids, bacteriophages ,transposons, integrative conjugative elements, IS (insertion sequence) elements, integrons, gene cassettes, and genomic islands. Since remnants of MGEs are frequently found adjacent to genes identified as horizontally transferred in their complete

genome sequences, plasmid, phage, or transposon-related sequences can be linked to a high proportion of horizontally transferred genes in many species (Ochman et al., 2000; Brüssow et al., 2004; Gal-Mor and Finlay, 2006). MGEs are crucial elements that encourage quick adaptation to evolving conditions, which in turn promotes bacterial diversification. Mechanisms that have evolved in MGEs have improved the possibility of gene transfer between organisms.

For instance, viruses and conjugative elements have developed incredibly effective methods for transferring genes into a target cell. Genes close to the insertion site in the host genome may have their functions changed by MGEs. The disruption or inactivation of genes at the location of insertion is one example of these changes. On the other hand, the insertional mutagenesis caused by MGEs can also benefit the host by providing regulatory sequences, fixing double-stranded DNA breaks, or causing genome reorganization and diversification. Through genetic hijacking, HGT of MGEs can result in the transfer of additional genes. For instance, MGEs are the main means of dispersing pathogenic determinants, antibiotic genes, and biodegradation processes (de la Cruz and Davies, 2000; Top and Springael, 2003; Smets and Barkay, 2005; Gal-Mor and Finlay, 2006; Larraín-Linton et al., 2006).Bacterial populations can now inhabit entirely new niches through HGT (Burrus and Waldor, 2004).

1.9.4 Horizontal gene transfer

The stable transfer of genetic material from one organism to another without reproduction is known as horizontal gene transfer, or HGT. Although opinions on how important HGT is have fluctuated over the years (Doolittle et al., 2003), the majority of researchers today believe that HGT plays a significant role in bacterial evolution, if not the primary role. Three main mechanisms underlie horizontal gene transfer (HGT) between bacteria: conjugation (gene transfer via plasmids or conjugative and integrated elements), transduction (gene transfer mediated by bacteriophages), and transformation (the uptake of free DNA).

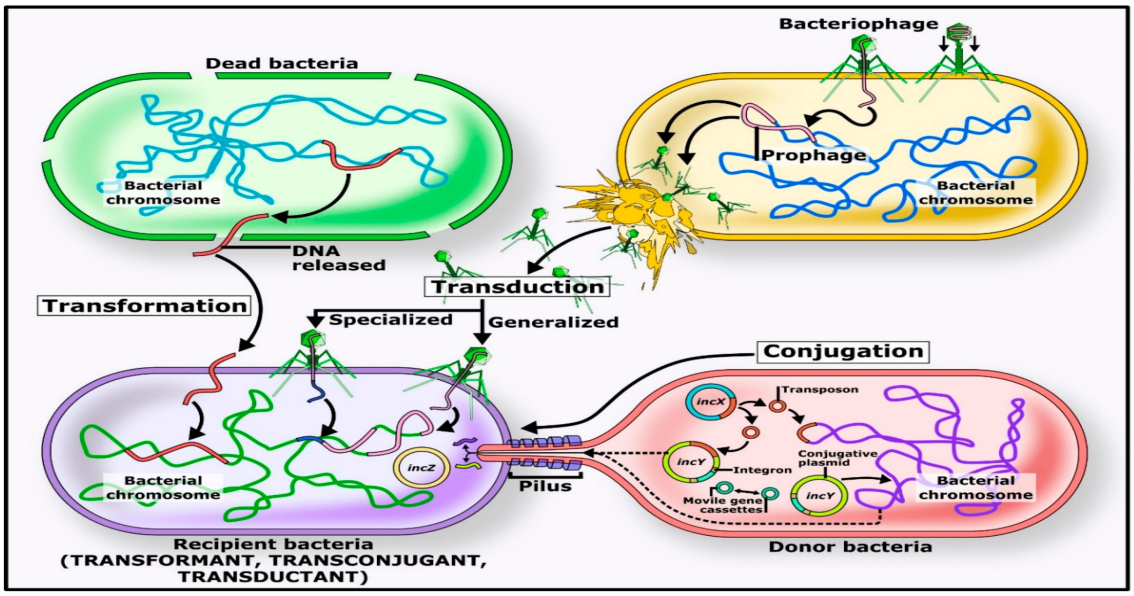


Figure 1.1: Mechanism of Horizontal Gene Transfer

(Lofston et al,2016)

1.9.5 Natural transformation

The widespread understanding is that natural transformation occurs when competent bacteria take up free DNA (Lorenz and Wackernagel, 1994; Dubnau, 1999, Chen and Dubnau, 2004). A physiological state that is genetically programmed and allows for the effective uptake of macromolecular DNA is known as natural competence.

Natural transformation is a highly controlled process involving over a dozen proteins and complex machinery. It appears that not all isolates from the same species share transformability, and among transformable isolates of the same species, transformation frequencies can differ by up to four orders of magnitude (Sikorski et al., 2002; Maamar and Dubnau, 2005). According to Dubnau (1999), the uptake of DNA can be used as a supply of nutrients, for DNA repair, or for genetic development. Following uptake of DNA, an autonomously replicating element may arise, homologous recombination, or homology-facilitated illegitimate recombination can be used to integrate the DNA into the bacterial genome (de Vries and Wackernagel, 2002). The absence of homologous sequences and replication origins were major obstacles to HGT via transformation (Thomas and Nielsen, 2005). Additionally, several bacteria lacking genes implicated in natural competence have been shown to undergo spontaneous transformation or transformation by lightning (Cérémonie et al., 2004, 2006). Through the usage of DNA found in their environment, competent bacteria can produce genetic variety through the method of gene transfer provided by natural transformation (Dubnau, 1999; Nielsen et al., 2000). The availability of free DNA, the growth of competence, and the incorporation and stable integration or independent replication of the acquired DNA are the prerequisites for natural transformation. However, little is known about the significance of spontaneous transformation in many environmental conditions that support bacteria's ability to adapt. In bacterial biofilms, transformation might be essential for the production, upkeep, and transfer of genes (Molin and Tolker-Nielsen,

2003; Petersen et al., 2005). The persistence of free DNA and the capacity of various bacterial species to become competent and take up free DNA under environmental settings are two additional features of natural transformation in the environment that have received the most research attention.

1.9.7 Transduction

Non-viral DNA can be transferred from an infected host bacteria to a new host by the technique of transduction, which involves infectious or non-infectious virus particles. The host's DNA is unintentionally packed into the vacant phage head during the production of the phage particle. According to Brüssow et al. (2004), defective phage particles that are liberated from lysed host cells may adhere to new host cells and transfer the DNA contained in the capsid to the new host. However, offspring phage production is prohibited by the incomplete phage genome. It is possible for the injected bacterial DNA to be integrated into the recipient genome through mechanisms that often call for the presence of integrases or homologous DNA sequences.

Gene transfer by bacteriophage mediators can be classified as generalized or specialized transduction based on their phenotype. Specialized transducing phages are temperate phages that transfer only specific genes that are closer to the phage integration site, whereas generalized transducing phages can be either temperate or virulent phages that carry any type of host DNA regardless of location. The ability of the bacterial hosts to acquire fitness benefits—such as protection against lytic infections—is a crucial feature of prophage acquisition. Moreover, phages are essential for the development of bacterial diseases.

1.9.8 Plasmid

Plasmids play a crucial role in the build-up and dissemination of ARGs, primarily in Gram-negative bacteria. They also play a part in the acquisition of resistance to the majority of

antibiotic classes, such as quinolones, aminoglycosides, tetracyclines, trimethoprim, sulfonamides, macrolides, and polymyxins (Carattoli, 2013; Shintani et al., 2015). Circular or linear plasmids are persistent replicating organisms with intricate replication machinery (Shintani et al., 2015). Plasmids often replicate autonomously and are physically different from the main bacterial chromosome; nonetheless, the majority of the resources needed for replication are provided by the host

The purposes of plasmids

The primary purpose of a plasmid is to spread antibiotic-resistant genes throughout the entire body of an animal or human. Plasmids can also carry genes that increase the pathogenicity of bacteria that cause diseases like tetanus, anthrax, and others. These genes are involved in metabolic processes and aid in the digestion of environmental pollutants. Additionally, plasmids can produce antibacterial proteins.

Plasmids can also give bacteria the capacity to break down resistant chemical compounds or fix elemental nitrogen, which is essential when nutrients are limited (Lipps et al. 2008).

Types of plasmid

Bacterial conjugation involves resistance plasmids. Typically, they possess the genes responsible for antibiotic resistance. Additionally, the genes responsible for producing the sex pilli's conjugation are encoded by them. Transferring the resistant plasmid from a donor bacterium to the recipient bacteria is the primary function of the pilli during conjugation. The other bacteria develop resistance in the same way.

Degradative plasmids: The dead organic stuff from dead animals or plants can be broken down or digested by this kind of plasmid. Through the process of biosynthesis, they generate energy from this organic matter and recycle it.

Fertility Plasmids: These plasmids include trans-genes that are necessary for conjugation. They aid in the genetic material's transferring across bacteria.

COL plasmids: These plasmids generate the antibiotics that, by remaining within the host bacterial cell, aid in the destruction of other dangerous bacterial strains. Colicin virulence plasmids, another name for the antibiotics, have the capacity to turn bacteria into pathogens. These are the genes that give rise to the illness.

1.9.9Targeting MGEs to Combat Antibiotic Resistance

Bacteria are becoming resistant to all currently prescribed antibiotics, making the development of new, more effective medicines or other therapeutic techniques imperative (Seal et al., 2018). In the environmental and clinical domains, MGEs are critical for the growth and dissemination of ARGs, especially those that carry resistant plasmids, transposons, and integrons. Therefore, the hypothesis that eliminating these MGEs could be one method of lowering AMR is strongly supported by the available data. In medicine, "curing" refers to a range of clinical interventions meant to restore an unstable system (Kennedy, 1981; Dow, 1990). "Curing" refers to the process of removing ARGs from bacterial populations; the materials used to accomplish this are called "curing agents." Since plasmids contain the bulk of ARGs and virulence factors, removing them has been associated with the "curing" process since 1971 (Bouanchaud and Chabbert, 1971). Over the past 50 years, tests have been done on antibacterial compounds such as detergents, biocides, intercalating agents, and nanoparticles; treatments based on bacteriophages and microbiota; and the CRISPR system for removing resistance in plasmids (Buckner et al., 2018).

1.9.9.1 Plasmid curing agents

Plasmid curing is the process of removing a plasmid from its host cell by use of a physical, chemical, or biological agent. The physical procedure entails subjecting the cells to extreme heat, light, microwave radiation, and ultraviolet radiation. Acriflavine, acridine orange, sodium dodecyl sulfate, novobiocin, and ethidium bromide are the chemical approaches

employed, while various plant extracts constitute the biological agent. A lot of substances have demonstrated some plasmid curing ability. Detergents, biocides, DNA intercalating agents, antibiotics (such as rifampicin, quinolones, and aminocoumarins), ascorbic acid, psychiatric medications (such as chlorpromazine), and compounds derived from plants are among them. These substances' relative efficacy varies widely and is contingent upon the plasmid, bacterial strain, and growing environment. Plasmid curing chemicals exhibit different mechanisms of action. Several substances interfere with plasmid replication either by integrating into the DNA (as in the case of intercalating agents and chlorpromazine), breaking DNA (as in the case of ascorbic acid), or affecting plasmid supercoiling (as in the case of aminocoumarins and quinolones).There are still certain curative drugs whose exact mechanisms of action are unclear. It is possible to hypothesize that plasmid curative drugs could also target plasmid segregation by raising the fitness cost linked to plasmid carrying or by impeding equitable distribution across daughter cells.

1.9.9.2 DNA intercalating agents

Intercalating dyes that act as curing agents include quinacrine, ethidium bromide, acridine orange, and acriflavine (Litake, 2022). The suppression of plasmid replication is the mechanism of action of intercalating agents. According to Crémieux et al. (1995), the dyes are usually flat, horizontal substances that can fit in between the stacked base pairs of the plasmid's DNA. The dyes' ability to insert themselves into the DNA double helix and stop plasmid DNA replication, ultimately resulting in the loss of plasmids in the microbial cell, makes this intercalation achievable (Howe et al., 2007). An intercalating agent's maximal effective concentration might differ greatly, ranging from 100 to 1000 times (Haque, 2017).

This is dependent upon the curing substance's efficacy, the species being treated, and the curing agent's mode of action. Doses of 0.05 to 0.2 mg/ml of acridine orange have been

administered (Letchumanan et al., 2015). As a benefit, DNA intercalating agents can be applied to various DNA sequencing techniques as well as DNA staining, visualization, quantification, and identification under a microscope in addition to being utilized to cure plasmid DNA (Li and Sancar, 2020). But problems occur when they try to employ them in live cells or in therapeutic settings because of their mutagenicity, cytotoxicity, non-specific binding, and environmental concerns (Sirajuddin et al., 2013).. Bacteroides fragilis and B. thetaiotaomicron (Rotimi, Duerden and Hafiz 1981), *S. aureus* (Jetten and Vogels 1973; Ersfeld-Dressen, Sahl and Brandis 1984), Lactobacillus plantarum (Adeyemo and Onilude 2015), Vibrio parahaemolyticus (Letchumanan et al. 2015), and E. coli were all cured by acridine orange. It was discovered that ethidium bromide could remove plasmids from several strains of Escherichia coli and *S. aureus* in the late 1960s and early 1970s (Bouanchaud, Scavizzi and Chabbert 1969; Rubin and Rosenblum 1971). The loss of plasmid-encoded staphylococcin production in Staphylococcus species was caused by acriflavine, ethidium bromide, and acridine orange (Jetten and Vogels 1973; Ersfeld-Dressen, Sahl, and Brandis 1984). Nevertheless, strains that developed resistance to acriflavine also developed resistance to its curing effects. Because DNA intercalating agents are potent mutagens that are also very toxic and carcinogenic, there aren't many practical applications for them. Any possible advantage of plasmid healing is greatly outweighed by the risks associated with utilizing such medicines. Furthermore, the usage of such compounds may favor the overexpression of bacterial efflux pumps, which may result in MDR, since many intercalating agents are substrates of these pumps (Piddock 2006). Nevertheless, in a lab setting, these substances can still be helpful in treating plasmid strains (Coleri et al. 2004; Mesas, Rodriguez and Alegre 2004; Chin et al. 2005; Raja and Selvam 2009; Zaman, Pasha and Akhter 2010; Adeyemo and Onilude 2015; Pulcrano et al. 2016).

1.9.9.3 Plant-derived compounds

Traditional medicine is the source of several thoroughly researched plant extracts. The tropical/subtropical Plumbago species' roots are the source of plubagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a yellow dye (Patwardhan et al. 2015). According to reports, plumbagin possesses antibacterial, antifungal, and anticancer properties (Padhye et al., 2012; Tyagi and Menghani, 2014). Plumbagin successfully removed the RP4 plasmid and a conjugative MDR plasmid from E. coli (Lakhmi, Padma, and Polasa 1987; Bharathi and Polasa 1991). By lowering the amount of plasmid copies and lessening the harmful effects of plasmid loss, plubagin eradicated plasmids from E. coli (Lakshmi and Thomas 1996). When combined, plant-derived compounds have the potential to cure plasmids in vitro; however, further study is required to establish the range of activity, pinpoint the active ingredients, ascertain any potential toxicity, and assess the efficiency of the compounds in vivo.

1.9.9.4 Dna gyrase inhibitors

The enzyme DNA gyrase is present in bacterial cells and is in charge of the supercoiling of DNA. Transcription, DNA replication, and other biological functions depend on supercoiling. DNA gyrase inhibitors precisely target DNA gyrase and block its function. Examples of these include novobiocin and quinolone antibiotics (e.g., ciprofloxacin) (Khan et al., 2018). According to Bush et al. (2015), plasmids usually possess systems for dividing into daughter cells through the use of the DNA gyrase enzyme. This guarantees that a copy of the plasmid is inherited by both daughter cells. The inhibition of DNA gyrase results in the disruption of plasmid replication. Consequently, during cell division, plasmids may be segregated unevenly, which could result in the plasmid being lost in one of the daughter cells (Higgins et al., 2015). Bacteria may become more vulnerable to drugs or other environmental challenges as a result of this loss. The exact experimental settings, as well as the features of the bacterial strain and

plasmid under consideration, can affect the concentration of DNA gyrase inhibitors employed to cure plasmids (Kamruzzaman and Iredell 2019). According to Nuonming et al. (2018), the usual concentrations of ciprofloxacin and novobiocin are 1 µg/ml to 10 µg/ml or even higher. While plasmids are successfully removed from bacterial populations using DNA gyrase inhibitors for plasmid curing (Riber et al., 2016), this method also restores antibiotic susceptibility and facilitates research. Its drawbacks include variable efficacy, cytotoxicity, the emergence of resistant isolates, and off-target effects, which occur when the plasmid DNA in question is not attacked.

1.9.9.5 Rifampicin

the enzyme RNA polymerase, which is in charge of converting DNA to RNA during gene expression, is the main target of rifampicin. By creating RNA strands complementary to the DNA template, RNA polymerase attaches to the promoter regions of genes to start transcription (Lilic et al., 2020). Rifampicin impedes the synthesis of RNA from DNA templates by binding to RNA polymerase. This inhibition affects chromosomal gene transcription as well as plasmid gene transcription, including plasmid replication and maintenance genes (Sutormin et al., 2022). Rifampicin concentrations for plasmid cure typically range from roughly 2 to 7.5µg/ml (Mitchell and Kenworthy, 1977). On the other hand, the ideal concentration for every unique plasmid and bacterial strain should be ascertained empirically. Plasmid curing using rifampicin is an effective way to get rid of plasmids, however there are some drawbacks such as cytotoxicity, global transcription effects, resistance emergence, and unreliable outcomes (Roy et al., 2021).

1.9.9.6 Sodium Dodecyl Sulfate

Since some plasmid-containing cells have plasmid-specific pili on their cell surfaces, it is thought that these cells are more vulnerable to sodium dodecyl sulfate. Certain plasmids can

be cured with sodium dodecyl sulfate (Caro et al., 1984). By attaching to hydrophobic areas of proteins and rupturing their three-dimensional structures, it denatures proteins (Moriyama and Takeda, 1999). Many of these proteins, including DNA polymerases, helicases, and primases, are essential for DNA replication and need the right folding and structure in order to work. These proteins can be denaturated with sodium dodecyl sulphate to reduce their activity, which will impede the replication of DNA in plasmids and chromosomes. Although sodium dodecyl sulphate has been widely utilized for protein denaturation, cell lysis, and solubilizing biomolecules in laboratory operations, there have been few investigations conducted on its usage for plasmid curing (Di Carlo et al., 2005). Although concentrations as high as 10% and 0.002% have been reported to be effective against S. aureus, not much is known about the range of doses needed for cure (Sonstein, and Baldwin, 1972). Sodium dodecyl sulfate (SDS) has the advantage of being useful for cell lysis, electrophoresis, and protein solubilization and denaturation. Limitations include the possibility of cytotoxicity at high dosages and interference with specific assays (Newby, 2000).

1.9.9.7 Mitomycin C

A substance known to damage DNA, mitomycin C creates covalent crosslinks between DNA strands. Inter-strand crosslinks are formed when it attaches to the DNA molecule, physically joining the two DNA strands. RNA polymerase is unable to start transcription because of the added DNA cross-linkage (Ross and Siegel, 2021). The cells initiate repair pathways, such as homologous recombination or nucleotide excision repair, to repair the DNA damage when mitomycin C causes DNA crosslinks and replication issues (Dronkert and Kanaar, 2001). The bacterial cells lose the plasmid as a result of DNA repair and recombination activities, which also eliminates the resistance genes the plasmids in the bacterium carried. The particular bacterial strain, plasmid, and experimental settings can all affect the range of effective

Mitomycin C doses for plasmid cure. According to Dörr et al. (2009), it is commonly applied at comparatively modest quantities,

usually between 0.1 and 1 microgram per milliliter (µg/mL) of bacterial growth. Lower dosages might work well for bacterial isolates or less resistant plasmids that are more vulnerable to the antibiotic's effects. When dealing with highly stable plasmids or bacterial isolates with strong resistance to Mitomycin C, higher concentrations in the range of 10 to 100 µg/mL or even higher may be required to achieve plasmid curing. Concentrations of Mitomycin C in the range of 1 to 10 µg/mL are used for plasmid curing in certain bacterial isolates or with plasmids that have moderate resistance (Jacoby, 1974). Mitomycin C has some benefits for plasmid cure, such as its ability to break DNA and encourage plasmid removal; nevertheless, it also has some drawbacks, such as the requirement to determine the ideal dosage through empirical means and the possibility of cytotoxicity to the host cells. When plasmid loss results in the reversal of antibiotic resistance, it may be advantageous in therapeutic settings (Andersson and Hughes, 2010). It can raise the likelihood of a therapeutic result and improve the efficacy of treatment with microbial agents. To summarize, the deletion of a plasmid can increase the susceptibility of bacteria to antibiotics under test, while reducing their pathogenicity and virulence. It is an important phenomenon that should be taken into account while studying bacterial evolution as well as in clinical settings. (Rasko and others, 2005).

1.9.9.9 Factors that affect the efficiency of Plasmid curing agents

Certain plasmid curing agents require specific utilization mechanisms that are only functional in particular growing environments. Furthermore, some plasmids can only replicate at specific temperatures due to temperature-sensitive replication origins. The longer the plasmid curing agent has to function, the slower the bacteria grow. This is because there is a decreased chance of plasmid loss in bacteria that are growing and dividing quickly. Slow-

growing bacteria may find it more difficult to heal from any damage that the plasmid curing agents have caused.

Plasmid copy number The effectiveness of a plasmid curative agent can be influenced by the quantity of plasmid copies in a cell. It could be challenging to eliminate a plasmid if there are more copies of it.

The plasmid's characteristics and function can affect the efficiency of plasmid curing agent because plasmids come in a variety of forms, and the kind of plasmid used can influence how effectively a plasmid curing agent functions.

The agent's mode of action; When bacteria are rapidly replicating their DNA, like during their logarithmic phase of growth, Plasmid curing agent that impair plasmid replication may be more effective.

The specific genes that the plasmid carries can affect the efficiency of plasmid curing agents. Certain plasmids include genes necessary for the cell to survive, whereas others do not. It can be more difficult for plasmid curing agent to eliminate plasmids containing essential genes.

1.9.9.1 Justification of study

Understanding the function of plasmids and the mechanisms of action of plasmid curative agents may facilitate the creation of novel approaches to counteract antibiotic resistance. This study examines the plasmid profile of bacterial isolates that were taken from ear, nose, and throat clinic patients who had ear infections. Future research on this subject may benefit from the findings of this study, which will also assist in identifying the factors that influence the effectiveness of plasmid curing agents.

1.9.9.2 Aim and Objectives of the study

The purpose of this investigation was to examine the plasmid profile of bacteria isolates taken from patients who had ear infections as well as the demographics of those patients. Specific objectives were:

- To evaluate the trends, demographic distribution and comorbidities associated with ear infection at the study centre. Ear,Nose and Throat clinic of University of Benin Teaching Hospital.
- To isolate aerobic and anaerobic bacteria associated with ear infection at the study centre.
- To identify and confirm the presence of resistant isolates through phenotypic (antibiotic susceptibility testing) testing focusing on antibiotic resistance.
- To determine the antibiotic susceptibility profiles of the bacterial isolates using standard disc diffusion before and after curing.
- To design and execute plasmid curing experiments to selectively eliminate plasmids from the resistant isolates.
- To compare the antimicrobial resistance patterns of cured and uncured isolates to assess the role of plasmids in conveying resistance.

CHAPTER TWO

METHOD

2.1 Reagents and Chemicals

Glucose, lactose, maltose, mannitol, sucrose, galactose, hydrogen peroxide, Kovac's reagent, Crystal violet, Lugols's Iodine and oil immersion, Methylated spirit (SPC Co. Ltd. Nigeria), Shaker bath, Acetone, ethydium bromide, decolorizer, Safranin, Sodium pellets, Pyrogallol crystals (Loba Chem Pvt Ltd. India), Levofloxacin (20ug), cefotaxime (10ug), sparfloxacin (10ug), ciprofloxacin (30ug), amoxicillin (30ug), augmentin (10ug), gentamicin (30ug), pefloxacin (30ug), ofloxacin (10ug), azithromycin (12ug).

2.2 Culture Media

Sodium thioglycolate, Mueller Hinton agar, blood agar, nutrient agar, Mac conkey agar, nutrient broth, manitol salt agar,

2.3 Equipment

Hot air oven and incubator, autoclave, compound light microscope, refrigerator (Thermocool, UK), digital weighing scale, anaerobic culture chamber (DFT Techlogies, Chennai-India).

2.4 Glassware and other apparatus

Beakers, conical flasks, bottles (MacCartney, Universal and Bijoux), measuring cylinders. glass stirrer, glass slides, Petri dishes and Pasteur pipette (All glass wares were products of Pyrex, England), Sterile syringes, Bunsen burner, cotton wool, Pooled Human plasma, sterile swab sticks Surgical gloves, surgical blades, slide (Micropoint, China), foil paper, micropipette (OEM Manufacturers) and anaerobic Chamber (Mcintosh and Filde's), wire loop.

2.5 Study Design

The study was carried out at the Ear, nose and throat(ENT) Clinic of the University of Benin Teaching Hospital, Benin city Edo state. a tertiary health care centre that has an ear, nose and throat clinic attached to it that attends to both in-patient and out-patient.

2.6 Ethical Considerations

Informed consent was sought and obtained from participants, while assent was obtained from patients aged 18 years and below, following informed consent from their parents, after obtaining ethical approval from the Health Research Ethics Committee of the University of Benin Teaching hospital (PROTOCOL NUMBER ADM/E22/A/VOL. VII/14838152180).

Participants were duly interviewed and briefed on the scope of the study which included obtaining demographic data and medical history of the participants, collection of sample as swab from the nostril for microbiological analysis using sterile swab. Participants were assured of the absence of risks and harm associated with participating in the study and that they have the right to opt-out if they do not feel comfortable with the process before obtaining their informed consent.

The principle of voluntary participation, maintenance of anonymity, and confidentiality was maintained throughout the study. Participants were given the right to decide whether to participate, withdraw at any point, or decline to provide information on unclear points. Information provided by participants was treated confidentially, with no request for their address in the questionnaire.

2.7 Data Collection

Each study participant provided numerous details via a semi-structured questionnaire. Sociodemographic details included names withheld for privacy, gender, history of alcohol and tobacco use, medication history, presence of underlying or hereditary health conditions

(such as diabetes, hypertension, cardiovascular disease, etc.), frequency of ear treatment appointments, history of otitis externa or media, use of cotton buds or other sharp objects to clean the ear, swimming history, and ear piercings.

2.8 Population of Study, exclusion and inclusion criteria.

All eligible study participants, regardless of age, who came to the clinics with a clinical diagnosis of an ear infection from a qualified medical professional were included in the study. The diagnosis should be based on accepted diagnostic criteria and may include symptoms like discharge, ear, jaw, or reduced hearing, fever, and other related symptoms. voluntarily expressing their willingness to participate in the study for the specified study period (January 2024 to April 2024), participants must also give informed consent. To avoid adverse drug reactions, research participants who have a history of allergy to any of the test substances—such as antibiotics—will not be allowed to continue.

2.9 Specimen collection

A minimum of 127 specimens from the study center were assessed for the study as described by (Kumurya et al,2010). The period of collecting data for this project was January 2024–April 2024.A Sterile swab stick was gently inserted into the external ear canal 2cm deep by rotating the swab stick clockwise and anti-clockwise in order to ensure that the swab stick comes in contact with the tympanic membrane. A needle may be utilized to aspirate the fluid for analysis. After collection, the swab stick will be properly labeled and put in 20 milliliters of thioglycolate broth to preserve it. .All specimens were transported within 2 hours to the Department of Pharmaceutical Microbiology laboratory for further microbiological investigations.

2.10 Sample size determination

Sample size was calculated using a formula by (Kish and Lesley,1979); $n=Z^2p(1-p)/d^2$. Where; Z =Z score for 95% confidence interval=1.96, P=Prevalence, d= acceptable error (5%). Thirteen percent(13%) prevalence of ear infection was used.

$$N=\frac{(1.96^{2)} \times 0.16(1-0.16)}{(0.05)^2} = 207$$

Sample size of 207 was obtained after substituting the above parameters. Due to time constraint during the stipulated approved period of study by the research committee of UBTH, only 127 swab samples were collected and used for this study.

2.11 Laboratory investigation

2.11.1 Standardization of innoculum:

In a clear sample bottle, 0.5 McFarland standard was prepared using 1% barium sulfate and 1% sulfuric acid to form barium sulfate. Under Aseptic conditions, isolates were innoculated into a clear sample bottle containing distilled water using a sterile wire loop. The suspension was incubated at 37°c. The McFarland standard and the suspension tubes were held side by side against a white background with adequate lighting. The turbidity of the suspension was adjusted by adding more culture or saline until it visually matched the turbidity of the standard. The suspension was then mixed thoroughly to ensure uniform distribution of the micro orgamism (McFarland, J,1907)

2.11.2 Preparation of media:

2.11.2.1 Mac Conkey agar

Mac conkey agar was prepared according to the manufacturer's instruction. 48.55 grams of MacConkey agar powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder,

sufficient distilled water was added into the flask to make it up to 1000mL. Heat was applied to the mixture while stirring to dissolve the agar completely. The agar was sterilized in the autoclave at 121°C for 15 minutes After autoclaving, the agar was allowed to cool, then 20mL of molten agar was poured into the petri dish, the molten agar in the petri dish was allowed to set, then dried in the oven 50°C for 10 minutes. The plates were then labelled with the ID of the patient.

2.11.2.2 Manitol salt agar

Manitol salt agar was prepared according to the manufacturer's instruction. 78grams of manitol salt agar powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL.Heat was applied to the mixture while stirring to dissolve the agar completely. The agar was sterilized in the autoclave at 121°C for 15 minutes After autoclaving, the agar was allowed to cool, then 20mL of molten agar was poured into the petri dish, the molten agar in the petri dish was allowed to set, then dried in the oven 50°C for 10 minutes. The plates were then labelled with the ID of the patient.

2.11.2.3 Mueller Hilton agar

Mueller Hilton agar was prepared according to the manufacturer's instruction. 40grams of Mueller Hilton agar powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL. Heat was applied to the mixture while stirring to dissolve the agar completely. The agar was sterilized in the autoclave at 121°C for 15 minutes After autoclaving, the agar was allowed to cool, then 20mL of molten agar was poured into the petri dish, the molten agar in the petri dish was

allowed to set, then dried in the oven 50°C for 10 minutes. The plates were then labelled using the ID of the patient

2.11.2.4 Nutrient agar

Nutrient agar was prepared according to the manufacturer's instruction. 28grams of nutrient agar powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL. Heat was applied to the mixture while stirring to dissolve the agar completely.The agar was sterilized in the autoclave at 121°C for 15 minutes After autoclaving, the agar was allowed to cool, then 20mL of molten agar was poured into the petri dish, the molten agar in the petri dish was allowed to set, then dried in the oven 50°C for 10 minutes. The plates were then labelled using the patient ID

2.11.2.5 Blood Agar

10mL of defribinated blood was added to 100mL of an already prepared molten nutrient agar to prepare 10% blood agar, the mixture was shaken gently. The molten blood agar was then poured into plates and allowed to set, the plates were then dried in the hot air oven at 50°c for 10 minutes. The plates were then labelled using the patient ID

2.11.2.6 Sodium thioglycolate broth

Sodium thioglycolate was prepared according to the manufacturer's instruction. 29grams of sodium thioglycolate powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL. Heat was applied to the mixture while stirring to dissolve the agar completely.The broth was sterilized in the

autoclave at 121°C for 15 minutes After autoclaving, the broth was allowed to cool, then 5mLof sodium thioglycolate was poured into bottles.

2.11.2.7 Nutrient broth

Nutrient broth was prepared according to the manufacturer's instruction. 13grams of nutrient broth powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL.Heat was applied to the mixture while stirring to dissolve the powder completely. The broth was sterilized in the autoclave at 121°C for 15 minutes After autoclaving, the broth was allowed to cool, and poured into sample bottles. The bottles were labelled with patient ID

2.11.2.8 Citrase agar

Simmons citrate agar was prepared according to the manufacturer's instruction. 24.28grams of Simmons citrate powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL. Heat was applied to the mixture while stirring to dissolve the agar completely .The agar was sterilized in the autoclave at 121°C for 15 minutes After autoclaving, the agar was allowed to cool, then 10mL of molten agar was poured into test tubes containing Durham tubes and tilted to give a distinct slant

2.11.2.9 Urea agar

Urea agar was prepared according to the manufacturer's instruction. 18.71grams of urea agar base powder was weighed accurately into a 1000mL conical flask, 500mL of distilled water was added into the flask and shaken to ensure even distribution of the powder, sufficient distilled water was added into the flask to make it up to 1000mL. Heat was applied to the mixture while stirring to dissolve the agar completely. The agar was sterilized in the

autoclave at 121°C for 15 minutes After autoclaving, 50 mL of sterile 40% urea solution was added to the preparation. It was mixed well and 10mL was poured into test tubes.

2.12 Characterization and Identification of Isolates

After incubation in sodium thioglycolate broth for 24 hours, the sample were sub-cultured into 10% blood agar plate which were appropriately labelled to prevent errors. Plates labelled aerobes were incubated for 24 hours at 37°c. For anaerobes, Buchner's method for anaerobiosis was employed, which involved the use of an anaerobic jar, pyrogallol crystals and sodium hydroxide. The bacteria culture obtained was sub-cultured on 10% blood agar and placed in an anaerobic jar which had 5g of pyrogallol at the base of it. 10ml of 4% sodium hydroxide was added to the pyrogallol crystals. The jar was sealed tightly and rocked continually to allow complete reaction (Ananthanarayan and Paniker, 2006).. This method created an anaerobic environment for the growth of anaerobic bacteria. This system was allowed to incubate for 72hours at 37°c.

Isolates were then subcultured into differential media, manitol salt agar and Mac conkey agar. They were incubated at 37°c for 24 hours. The isolates obtained were subject to standard biochemical tests which included

2.12.1 Catalase Test

A small amount of bacteria isolate was placed on a dried glass slide using a sterile innoculating wire loop, a drop of (3%) hydrogen peroxide was added. The slide was observed for the presence of bubble. If bubbles form rapidly, it indicates the presence of catalase activity.(Brock *et Al.,*1969)

2.12.2 Coagulase Test:

A drop of saline was put onto the slide. The saline drop was emulsified with the test organism using a wire loop,. A drop of plasma (rabbit plasma anticoagulated with EDTA was

placed on the inoculated saline drop, mixed well, then the slide is rocked gently for about 10 seconds. The slide was observed for clumping. The formation of clot meant the test was positive while the absence of clot formation meant the test was negative.(schleifer *et Al.,*1982*)*

2.12.3 Citrase Test

The bacterial culture was streaked onto an already prepared citrate agar slant and Incubated at 37°C for 24hours. The agar was then observed for growth and color change. Growth and a blue color indicated a positive test, meaning the organism can utilize citrate as a sole carbon source while no color change indicated a negative change.(Simmons,1926)

2.12.4 Urease Test

The urea agar slant was inniculated with the test organism and Incubated at typically 37°C for 24 hours. After the incubation period, The agar was observed for a color change. A pink to magenta color indicated a positive test result, indicating urease activity If there is no color change, the test result is negative, indicating the absence of urease activity.(Christensen,1946)

2.11.5 Oxidase Test

Oxidase test was carried out using a filter paper and Kovac's reagent. The organism was placed on the filter paper using a sterile wire loop and a drop of kovac's reagent was added directly on the innoculum.purple-blue color developing within 10-30 seconds indicates a positive result, indicated the presence of cytochrome c oxidase activity.no color change in the specified time indicated that the test result was negative, indicating the absence of cytochrome c oxidase activity.(Kovacs,1956)

2.11.6 Indole Test

The indole test was carried out using a sterilized test tube that contained tryptophan broth. The tubes containing the broth was innoculated with the isolates and incubated for 24 hours.0.5ml kovac's reagent was added to the broth culture and the culture was observed for

the formation of a red ring at the top of the broth. The presence of a red ring indicated a positive result and the absence of the red ring indicated a negative result(Kovacs ,1956).

The isolates were also subjected to Gram staining where to a standardized suspension of the organism heat-fixed to a glass slide, crystal violet was added, followed by iodine treatment, ethanol decolorization, and then a safranin counterstain to differentiate bacterial cells into either Gram-positive (purple) or Gram-negative (pink) based on cell wall characteristics.(Gram H.C, 1884)

The Gram staining, catalase, coagulase, indole, citrase, urease and oxidase tests carried out, were used to identify the isolates.

The results for each isolate were obtained and documented accurately.

2.12 Antimicrobial Susceptibility Test

Isolates were subcultured into already prepared nutrient broth and incubated for 24 hours, the isolates were then subjected to antimicrobial susceptibility testing using the disc diffusion method and agar dilution method. The selection of test antimicrobial agents was based on the antibiotics used in the treatment of rhinitis at the ear, nose and throat clinic, they included azithromycin, augmentin, amoxicillin, perfloxacin, ofloxacin,sparfloxacin, gentamicin, co-trimoxazole and ciprofloxacin. The antibiotics on the disc used for disc diffusion method included ciprofloxacin, augmentin, amoxicillin, perfloxacin, ofloxacin, azithromycin, levofloxacin, Sparfloxacin. Mueller Hilton agar was prepared by weighing 28gram of agar into 1000ml of distilled water, shaken and sterilized in the autoclave, the agar was left to cool slightly then 20ml was poured into the petri dish, allowed to set, then taken into the hot air oven to dry at a controlled temperature of about 50ºC for 30minutes with the lid separated from the plate and both inverted with their inner surfaces facing downwards. The positive control Petri dishes did not have antibiotic disc incorporated on the Mueller-Hinton agar plate,

while the negative control dishes contained a specific strength of a stock solution. For the disc diffusion test, a sterile swab stick was introduced into each standardized inoculum and used to evenly streak the entire surface of a prepared Mueller-Hinton agar plate and the plate was allowed to stand, the Antimicrobial disc was then placed on the plate and incubated for 24 hours. The inhibitory zone diameter around each disc was measured with a calibrated ruler and recorded.

2.12.1 Minimum Inhibitory Concentration

For minimum inhibitory concentration(MIC), Agar dilution was used .Antibiotics tested were augmentin(12.5ug,25ug,50ug,100ug),ciprofloxacin(0.5ug,1ug,2.5ug and5ug),cotrimoxazole(2.5ug,5ug,10ug,20ug)and amoxicillin (12.5ug,25ug,50ug,100ug). The varying concentrations of the antibiotics used were calculated for, weighed and dissolved in sterile distilled water. The molten Muller Hilton agar was mixed with varying volumes of the dissolved antibiotics then poured onto a plate and dried in the hot air oven at 50°C for 10 minutes. hence, each plate contained different concentrations of the antibiotics. The innoculum was then streaked on the plates using a sterile wire loop. The plates were incubated for 24 hours, the plates were then observed for growth. The plate with the lowest concentration of antibiotic to prevent the growth of micrograms was recorded as the minimum inhibitory concentration.

All plates were incubated at 37 °C for 24 hours in duplicates. Inhibition zone diameters and minimum inhibitory concentrations were obtained and appropriately recorded before and after treating with ethidium bromide. These were interpreted as susceptible or resistant by comparison with published guidelines for antimicrobial susceptibility testing for commonly occurring pathogens, as obtained from clinical isolates (EUCAST, 2015).

2.12.2 Plasmid Curing

Isolates were collected and treated with acridine orange in accordance with Brown's recommended protocol after initial antimicrobial testing (Brown, 2000). The recipe called for double strength nutritional broth, to which 0.1 mg/mL and 0.4 mg/mL of acridine orange were added. A volume of 20 µL from the bacterial culture that was left overnight was subcultured into 5.0 mL of the nutritional broth that contained acridine ethidium bromide. After that, the samples were incubated in a shaker bath with a 150 rpm revolution rate for 72 hours at 37 °C.

The isolates were sub-cultured onto Mueller-Hinton agar after the 72-hour incubation period, and the antimicrobial susceptibility testing was then carried out once more. The objective of this phase was to measure the inhibition zone diameters, as previously mentioned, in order to ascertain the cured isolates' susceptibility to antibiotics.

2.13 Data Analysis

The semi-structured questionnaire obtained six patient demographic characteristics, which were coded and entered into the Statistical Package for Social Sciences (SPSS) version 21.0 software (SPSS Inc Chicago IL USA). These included history of smoking, drinking, and using medications, as well as age, sex, and occupation. The software defined these variables as string variables. Furthermore, questions with multiple choices that described activities like swimming, ear cleaning routines, and ear piercings were also classified as string variables. The sizes of the inhibitory zones were measured and quantified before the EUCAST breakpoint was analyzed. The percentage frequencies of the patient's demographics, medication history, and the existence of underlying or genetic health disorders (such as diabetes, hypertension, cardiovascular disease, etc.) were reported using descriptive statistics. The t-test and chi-square were used to calculate the statistical differences between the variables. Group differences were deemed significant when the P value was less than 0.05.

Standard operating protocols were followed in the calculation of standard diagnostic indices, such as positive predictive diagnostic (Gram staining test) efficacies and quantitative antibiogram (IZD) values.

CHAPTER THREE

RESULTS

3.1a Association of Age with Patient Demographics

Table 3.1 showed the frequency distribution of patient demographics obtained during the course of the research. A total of 76 patients were involved in the study with a total number of 56 females (73.68%) of which the highest frequency of females (26 females) fell within the age range of 21-25years and 11males (14.47%) with the highest frequency recorded among male participants within >60years. The least encountered age range was 46-55years (2.7%) followed by patients within the range of 55-60years (3.94%).

3.1b Association of Gender with Patient Demographics

Table 3.1b illustrated the association of gender with patient medical history with the inclusion of statistical significance. The highest number of patients(28 patients accounting for 36.84% of total population) reported symptoms which persisted for 8 - 14days with females having more frequency compared to males and the least duration of symptoms experienced was <7days. Study showed that 50 patients(65.79%) had previous episodes of ear infection which warranted treatment. Pain in the ear was the most common symptom among both male and female(38.16%). Conversely, other symptoms such as pain in the ear, discharge, fever, and itching were frequent among female compared to males. The least symptom experienced was turning of the eye which was reported by only 1 male participants.75% of male and 92.86% of female had never swam, majority never drank alcohol prior to visiting the ear,nose and throat clinic. The left ear was the most affected accounting for 40.78% of the study population. 55% of the male were found to have cleaned their ears, Out of a total of 56 female patients studied, approximately half(46.4%) were observed to have cleaned their ears. A higher proportion of female patient in the study reported to have never cleaned their

ear(11%) as compared to male patients(15%). A total of 20 male patients and 49 female patients reported using cotton buds to clean the ears. Meanwhile,6 female patients reported using biro tips for cleaning their ear, and one female patient reported using a key.

Table 3.1a: Association of Age with Patient Demographics

		Male	**female**	**Total**	P –value (ci=95%)
age of patients	0-6years	2	1	3	<0.00
	7-15years	0	8	8	
	16-20years	2	7	9	
	21-25years	3	26	29	
	26-30years	0	4	4	
	31-35years	2	2	4	
	46-55years	0	3	3	
	56-60years	0	2	2	
	>60years	11	3	14	
Total		20	56	76	
smoking history	never smoked	13	52	65	0.001
	stopped smoking	7	3	10	
	still smoking	0	1	1	
Total		20	56	76	
alcohol history	never drank	6	45	51	0.537
	still drinking	6	10	16	
	stopped drinking	8	1	9	
Total		20	56	76	
Occupation	Employed	9	8	17	0.088
	self employed	2	4	6	
	Student	2	10	12	
	not employed	5	34	39	
	Retired	2	0	2	
Total		20	56	76	

Table 3.1b: Association of Gender with patient Medical History

Medical history		Male	female	Total	p-value cl=95%
duration of symptoms	<7days	7	15	22	0.440
	8-14days	5	23	28	
	>14days	8	18	26	
If yes, previous ear infection	Right ear	3	13	16	0.575
	Left ear	10	21	31	
	Both	7	22	29	
		20	56	76	
Previous ear infection	Yes	13	37	50	0.931
	No	7	19	26	
		20	56	76	
FREQUENCY OF SYMPTOMS					
Pain in the ear		6	23	29	0.186
Discharge		5	14	19	
Poor hearing		4	3	7	
Fever		1	7	8	
Itching		3	9	12	
Turning of the eyes		1	0	1	
		20	56	76	

3.2 Association of aerobic bacterial isolates with patient characteristics

Table 3.2. introduced the association of aerobic bacterial isolates with patient characteristics, however there were no statistically significant relationship: Participants within the age range of 21-25 years had more aerobic isolates, Female patients(73.33%)had more aerobic microbial isolates as compared with the male patients with 26.66 %. Patients without an occupation accounted for 49.33% of all aerobic isolates, making them the demographic with the highest prevalence of anaerobic infections. Conversely, the retired demographic had the least occurrence of aerobic isolates, with a relatively low percentage of 4%. Also, those with no history of chronic disease had more of these microbial isolates in their ear .

Conversely, subjective data from study participants showed that patients who never drank alcohol and never smoked had higher number of microbial load in their ear while those still drinking (≥ 2 times a day) had low microbial load in the ear. Among patients in the study, those who reported that they never swam presented with the highest incidence of aerobic isolates(88%),indicating that this activity may be associated with a lower prevalence of aerobic infections, on the other hand, patients who reported having stopped swimming had the lowest percentage of aerobic isolates(5.33%). Patients still swimming had the second highest percentage of aerobic isolate (6.66%). Ear cleaning practices were linked to the prevalence of aerobic infections, with the highest incidence among patient who reported cleaning their ears using cotton buds(90.67%).Similarly, the frequency of wearing earrings was associated with aerobic isolates, with patients who reported wearing earrings everyday having the highest prevalence(62.27%).patients who never clean their ears reported the lowest aerobic isolate(12%), with patients who wore earrings only for ceremonies showing the lowest incidence (4%).

3.1 Frequency Distribution of Aerobic Isolates

The prevalence of *Staphylococcus spp*. was the highest (49.33%) in microbial isolate obtained from study participants after aerobic incubation. *Pseudomonas* spp. followed with a prevalence of 25%, while *Enterobacteriaceae spp* had the lowest frequency at 1.33% as illustrated by Fig 3.1.

3.2 Frequency Distribution of Anaerobic Isolates

Among the four organisms isolated under anaerobic conditions, as depicted in Figure 3.2, *Pseudomonas spp.* continued to exhibit the highest occurrence rate at 47.06%. Following this, *Bacillus spp*. accounted for 26,47% of the isolates, while *Staphylococcus aureus* (17.65%) and *Klebsiella spp* (8.82%) displayed lower frequencies of occurrence.

Table 3.2: Association of Aerobic Isolates with Patient Characteristics

		Staph Aur	Staph Epid	Corn Bact	Bacil Cer	Bacil Sub	Kleb Pneu	Kleb Oxy	Entero Bacter	Pseud Aero	Total	P Value Cl=95%
sex of patients	Male	9	1	1	2	2	0	0	0	5	25	0.555
	Female	12	15	2	3	4	2	1	1	15	55	
Age of patients (years)	0-15	1	1	1	0	0	0	0	0	0	3	0.501
	7-15	0	4	0	0	2	0	0	0	2	8	
	16-20	3	2	1	0	0	0	0	0	3	9	
	21-25	10	4	1	2	1	2	0	1	8	29	
	26-30	1	0	0	1	0	0	1	0	1	4	
	31-35	1	1	0	0	2	0	0	0	0	4	
	46-55	1	0	0	0	0	0	0	0	1	2	
	56-60	0	2	0	0	0	0	0	0	1	3	
	>60	4	2	0	2	1	0	0	0	4	13	
Occupation	employed	4	5	0	1	3	0	0	0	4	17	0.S060
	self employed	1	1	0	1	0	0	1	0	2	6	
	Student	3	3	1	0	0	1	0	0	4	12	
	not employed	12	7	0	2	3	1	0	1	10	37	
	Retired	1	0	0	1	0	0	0	0	1	3	
smoking history	never smoked	18	15	3	3	5	2	1	1	16	64	0.977
	stopped smoking	3	1	0	2	1	0	0	0	3	10	
	still smoking	0	0	0	0	0	0	0	0	1	1	
alcohol history	never drank	13	13	3	2	4	2	2	1	13	51	0.555
	Stopped drinking	4	0	0	1	1	0	0	0	2	9	
	still drinking	4	3	0	2	1	1	0	0	5	15	

3.3 Association of anaerobic bacterial isolates with patient characteristics

Table 3.3 showed the association of Facultative anaerobic isolates and patient characteristics. More females had Facultative anaerobic isolates in their ear cavity compared to males. The highest prevailing organism was Pseudomonas aeruginosa in which female patients had 14(41.18%) and male patients had 2(5.88%). Same trend was observed in Klebsiella pneumonia. Interestingly, more females had a high prevalence of Staphylococcus aureus compared to male. Notably, the highest frequency of anaerobic isolates was obtained from patients within the age of 21-25years with Staphylococcus aureus being the most encountered organism within this age range. Investigation revealed that the highest microbial load obtained were from students who participated in the study with Pseudomonas aeruginosa being the most prevalent isolate while Pseudomonas aeruginosa was more prevalent among participants who were unemployed. Notably. Retired participants had the least number of isolates with a distribution Pseudomonas aeruginosa. Participants who never smoked or drank had the highest distribution of isolates with Staphylococcus aureus being the most prevalent for those who do not consume alcohol and Pseudomonas aeruginosa for participants who do not smoke.

Table 3.3 Association of Anaerobic Isolates with Patient Characteristics

		Isolates					
		STAPH AUREUS	BACIL SUB	KLEB PNEU	PSEUD AERO	TOTAL	P-Value Cl=95 %
sex of patients	Male	1	4	1	2	8	0.357
	female	5	5	2	14	26	
age of patients (years)	0-6	2	0	0	0	2	0.641
	7-15	0	2	0	2	4	
	16-20	1	0	0	3	4	
	21-25	2	2	1	4	9	
	26-30	0	0	0	2	2	
	31-35	0	1	0	1	2	
	46-55	0	0	0	1	1	
	56-60	0	0	1	1	2	
	>60	1	4	1	2	8	
Occupation	employed	0	4	1	4	9	0.642
	self employed	0	1	1	1	3	
	student	5	3	0	8	16	
	not employed	1	1	1	2	5	
	retired	0	0	0	1	1	
smoking history	never smoked	5	6	2	15	28	0.403
	stopped smoking	1	3	1	0	5	
	still smoking	0	0	0	1	1	
alcohol history	never drank	4	4	2	12	22	0.474
	still drinking	1	3	0	3	7	
	stopped drinking	1	2	1	1	5	

KEYWORDS; STAPH AUREUS= *Staphylococcus aureus*, KLEB PNEU=*klebsiella pneumonia*, BACIL SUB= *Bacillus subtilis*, PSEUDO AERO =*Psueudomonas aeruginosa*

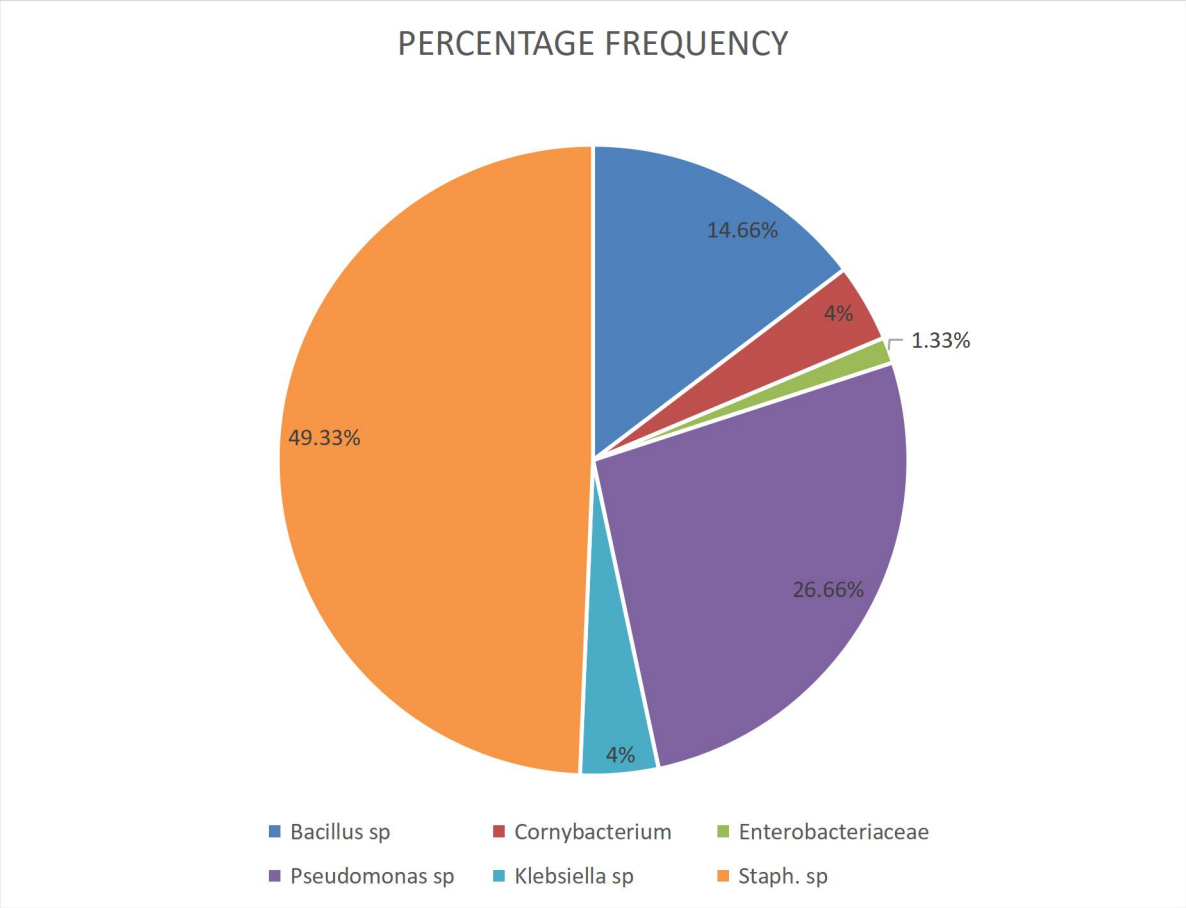


Figure 3.1: frequency distribution of Aerobic isolates

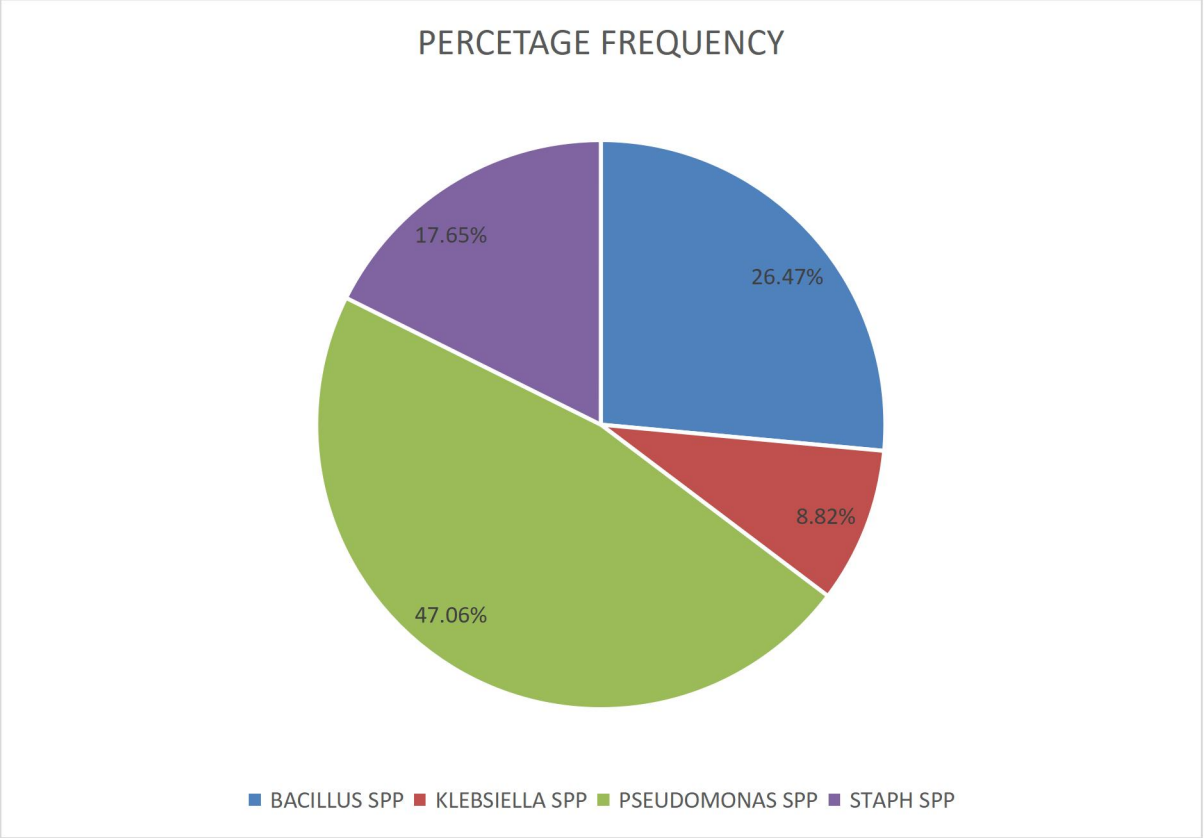


Figure 3.2: Frequency distribution of anaerobic isolate

3.4 Morphological and biochemical characteristics of anaerobic isolate

The following table provides a descriptive overview of the morphological and biochemical traits of isolates collected from patients diagnosed with ear infection.

Regarding their haemolytic properties, 5 isolates were classified as α-haemolytic, 22 as β-haemolytic, and 7 as ɣ-haemolytic.

Biochemical characterization revealed that all 34 isolates were catalase-positive. Among them, only Staphylococcus aureus isolates tested positive for coagulase (a total of 6 isolates). Furthermore, 16 isolates tested positive for oxidase, 0 for indole, 28 for citrate, and 9 for urease.

Gram-positive cocci were the most frequently encountered isolates (19), while Gram-negative bacilli were the least common, confirmed in 6 isolates.

3.5 Morphological and biochemical characteristics of aerobic isolate

The following table provides a descriptive overview of the morphological and biochemical traits of isolates collected from patients diagnosed with ear infection.

Regarding their haemolytic properties, 28 isolates were classified as α-haemolytic, 14 as β-haemolytic, and 33 as ɣ-haemolytic.

Biochemical characterization revealed that all 75 isolates were catalase-positive. Among them, only Staphylococcus aureus isolates tested positive for coagulase (a total of 37 isolates). Furthermore, 20 isolates tested positive for oxidase, 2 for indole, 72 for citrate, and 41 for urease.

Gram-positive cocci were the most frequently encountered isolates (37), followed by gram negative bacilli (24) while Gram-positive bacilli were the least common, confirmed in 14 isolates.

Table 3.4: Morphological And Biochemical Characteristics of anaerobic Isolates

Morphological TEST	Inference	Staph Aureus	Pseud Aero	Kleb Pneu	Bacil Sub	Total Isolate
Hemolysis characteristics	α haemolysis	0	3	1	1	5
	B haemolysis	1	12	2	7	22
	Ɣ haemolysis	5	1	0	1	7
Gram stain inference	GPC	0	16	3	0	19
	GNB	6	0	0	0	6
	GPB	0	0	0	9	9
Catalase test	POSITIVE	6	16	3	9	34
	NEGATIVE	0	0	0	0	
Coagulase test	POSITIVE	6	0	0	0	6
	NEGATIVE	0	16	3	9	28
Oxidase test	POSITIVE	0	16	0	0	16
	NEGATIVE	9	0	0	9	18
Citrate test	POSITIVE	0	16	3	9	28
	NEGATIVE	6	0	0	0	6
Indole test	POSITIVE	0	0	0	0	0
	NEGATIVE	6	16	3	9	34
Urease test	POSITIVE	6	0	3	0	9
	NEGATIVE	0	16	0	9	25

KEYWORDS; STAPH AUREUS= *Staphylococcus aureus*, KLEB PNEU=*klebsiella pneumonia*, BACIL SUB= *Bacillus subtilis*,PSEUDO AERO =*Psueudomonas aeruginosa*

Table 3.5: Morphological And Biochemical Characteristics of aerobic Isolates

Morphological Characteristic	Inference	Bacillus Spp	Corynybacterium Spp	Enterobacteriaceae Spp	Klebsiella Spp	Pseudomonas Spp	Staphylococcus Spp	Total Isolates
Hemolysis characteristics	Alpha	4	1	1	1	7	14	28
	Beta	4	2	0	0	4	4	14
	Gamma	3	0	0	2	9	19	33
Gram stain inference	GNB	0	0	1	3	20	0	24GNB
	GPC	0	0	0	0	0	37	37GPC
	GPB	11	3	0	0	0	0	14GPB
Catalase test	POSITIVE	11	3	1	3	20	37	75
	NEGATIVE	0	0	0	0	0	0	0
Coagulase test	POSITIVE	0	0	0	1	0	37	38
	NEGATIVE	11	3	1	2	20	0	37
Oxidase test	POSITIVE	0	0	0	0	20	0	20
	NEGATIVE	11	3	1	3	0	37	55
Citrate test	POSITIVE	11	0	1	3	20	37	72
	NEGATIVE	0	3	0	0	0	0	3
Indole test	POSITIVE	0	0	0	2	0	0	2
	NEGATIVE	11	3	1	1	20	37	73
Urease	POSITIVE	1	0	0	3	0	37	41
	NEGATIVE	10	3	1	0	20	0	34

Staphylococcus aureus, BAL= *Bacillus spp*, PSEUD= *Pseudomonas aeruginosa*, , KLEB= *Klebsiella pneumoniae*, +VE= Positive, -VE= Negative, GPC= Gram positive cocci, GPB= Gram positive bacilli, GNB= Gram negative bacilli

3.6 Antimicrobial Susceptibility Testing of Aerobes

Table 3.6 shows the antimicrobial susceptibility pattern associated with these clinically important isolates against various antimicrobial agents used in the treatment of ear infection. 75 of all total isolates were susceptible to the eliminating action of levofloxacin which is a classical example of antimicrobial agents which belong to the fluoroquinolone class. In general, majority of isolates obtained and tested were susceptible to fluoroquinolones. On the other hand, 75 isolates of total obtained isolates treated with amoxicillin which is an example of penicillin antibiotics were resistant to the eliminating action of the antibiotics.

Table 3.6: Antimicrobial Susceptibility Of Aerobes Using Disc Diffusion Method

Isolate	FQ	AMIGLY	CEF	MAC	PEN
Staphylococcus spp(n=37)	31 83.78%	29 78.38%	20 54.05%	26 70.27%	26 70.27%
Pseudomonas spp (n=20)	15 75%	13 65%	8 40%	14 70%	9 45%
Corynebacterium spp (n = 3)	3 100%	3 100%	3 100%	2 66.67%	2 66.67%
Bacillus spp (n = 11)	9 81.81%	10 90.90%	3 27%	6 54.54%	7 63.64%
Klebsiella spp (n = 3)	3 100%	2 66.67%	1 33.33%	3 100%	2 66.7%
Enterobacter spp (n = 1)	0 100%	0 0%	1 100%	0 0%	1 100%
Total (n=75)	61 81.33%	57 76%	35 46.66%	52 69.33%	47 62.66%

KEYWORD; FQ= Fluoroquinolones, AMIGLY= aminoglycoside, CEF= Cephalosporin, MAC= macrolide, AMOX/CLAV= Amoxicillin/clavulanic acid, AMOX= Amoxicillin,PEN =Penicillin, n= number of isolates

3.7 Antimicrobial Susceptibility Testing Of Facultative Anaerobes

Table 3.7 shows the antimicrobial susceptibility pattern associated with these clinically important isolates against various antimicrobial agents used in the treatment of ear infection. 24 of total isolates were susceptible to the eliminating action of fluoroquinolones with the highest susceptibility attributed to *Pseudomonas aeruginosa* (90% susceptibility). In general, majority of isolates obtained and tested were susceptible to fluoroquinolones. On the other hand, the highest frequency of resistance was recorded among penicillin particularly amoxicillin as reported showed a 25% susceptibility pattern across all isolates. The least susceptibility to penicillin was seen in *Klebsiella pneumonia* as only 11.1% of *Klebsiella pneumonia* isolate were susceptible to amoxicillin.

Table 3.7: Antimicrobial Susceptibility Pattern Of anaerobes Using Disc Diffusion Method

Isolate	FQ	AMIGLY	CEF	MAC	PEN
S. aureus (n=6)	5 83.3%	6 100%	4 16.67%	4 66.67%	3 50%
B. subtilis (n = 9)	9 100%	6 66.67%	5 55.55%	7 77.77%	7 77.77%
K. pneumonia (n = 3)	2 66.7%	1 33.33%	1 33.33%	1 33.3%	0 0%
P aeruginosa (n = 16)	15 93.75%	14 87.5%	6 37.5%	14 87.5%	9 56.25%
Total (n=34)	31 91.18%	27 79.41%	16 47.06%	26 76.47%	19 55.88%

3.8 frequency distribution of multi-drug resistant Aerobic isolates

Table 3.8 displayed the frequency of different isolates obtained during the course of investigation. The table showed that out of 75 clinically important isolates obtained, 13(17.33%) of these isolates were resistant to three or more classes of antimicrobial agents when tested using disc diffusion method. Among which, 50% of isolated *Klebsiella pneumonia* were multi-drug resistant, 9.52% of *Staphylococcus aureus* and 18.75% *of Staphylococcus epidermidis* were multi-drug resistant.35% of Pseudomonas aeruginosa were multidrug resistant. Interestingly, no multi-drug resistant isolate was observed for *Bacillus subtilis.*

Table3.8: Frequency of Multi-Drug Resistant Aerobic Isolates

	Frequency	Percent	MDR Strain	Percent
Staphylococcus aureus	21	28	2	9.52
Staphylococcus epidermidis	16	21.33	3	18.75
Cornybacterium spp	3	4	0	0
Bacillus cerus	5	6.66	0	0
Bacillus subtilis	6	8	0	0
Klebsiella pneumonia	2	2.67	1	50
Klebsiella oxytoca	1	1.33	0	0
Enterobacter aerogenes	1	1.33	0	0
Psueudomonas aeroginosa	20	26.67	7	35
	75	100.0	13	17.33

3.9 frequency distribution of multi-drug resistant anaerobic isolates

Table 3.9 displayed the frequency of different isolates obtained during the course of investigation. The table showed that out of 34 clinically important isolates obtained, 7(20.58%) of these isolates were resistant to three or more classes of antimicrobial agents when tested using disc diffusion method. Among which, 33.3% of isolated *Klebsiella pneumonia* were multi-drug resistant, 30% of total *Staphylococcus aureus* were multi-drug resistant. Interestingly, no multi-drug resistant isolate was observed for *Bacillus subtilis.*

Table 3.9: Frequency distribution of multidrug resistant anaerobic isolate

isolates	Frequency	Percent	MDR Strain	Percent
Staphylococcus aureus	6	17.65	1	16.6
Bacillus subtilis	9	26.47	0	0
Klebsiella pneumonia	3	8.82	3	100
Pseudomonas aeroginosa	16	47.06	3	18.75
Total	34	100.0	7	20.59

3.10 Plasmid Curing of Aerobic Isolates Using ethidium bromide

Table 3.10 showed the pattern of multi-drug resistance displayed by various isolates pre-curing and post curing using 0.1mg/mL and 0.4mg/mL Ethidium bromide. Notably, from the multi-drug resistant Staphylococcus isolates, 6 isolates had a common resistance to penicillin antibiotics. After curing, which 0% of these resistant isolates lost their resistance.The least Resistance displayed in *Pseudomonas aeruginosa* was attributed to the penicillin and cephalosporins. This resistance was lost upon curing as all resistant Staphylococcus aureus became susceptible to the killing action of fluoroquinolones.

Staphylococcus aureus recorded the highest resistance across all antimicrobial agents used with resistance to aminoglycosides, penicillin and macrolide being the most prevalent. Post-curing, 100% susceptibility was attributed to aminoglycosides, macrolides and cephalosporins. Interestingly, all isolates initially resistant to fluoroquinolones became susceptible to its antimicrobial action after treatment with ethidium bromide. Interestingly, resistance to penicillin which was persistent after supplementation with 0.1mg/mL ethidium bromide was conferred when the concentration was increased to 0.4mg/mL.

Table 3.10: Plasmid Curing of Aerobic Isolates Using ethidium bromide

ISOLATE			FQ	AMIGLY	CEF	MAC	PEN AMOX/ CLAV	PEN AMOX
S. aureus N=2	UNCURED		2	2	2	2	2	2
	CURED	0.1EB	1	0	0	0	0	0
		0.4EB	1	0	0	1	0	0
	% CURED		50%	0%	0%	50%	0%	0%
P. aeruginosa N=7	UNCURED		6	6	7	6	7	7
	CURED	0.1EB	3	1	0	2	0	0
		0.4EB	1	1	0	1	0	0
	% CURED		50%	16.67%	0%	16.67%	0%	0%
s.epidermis N=3	UNCURED		3	3	2	3	3	3
	CURED	0.1EB	1	1	1	1	0	0
		0.4EB	1	1	1	0	0	0
	% CURED		33.33%	33.33%	50%	33.3%	0%	0%
K. pneumonia N=1	UNCURED		1	1	0	0	1	1
	CURED	0.1EB	0	1	0	1	0	0
		0.4EB	1	0	0	1	0	0
	% CURED		100%	100%	0%	0%	0%	0%

3.11 Plasmid Curing Of Anaerobic Isolates Using ethidium bromide

Table 3.11 showed the pattern of multi-drug resistance displayed by various isolates pre-curing and post curing using 0.1mg/mL and 0.4mg/mL ethidium bromide. Notably, from the 3 multi-drug resistant *Pseudomonas aeruginosa* isolates, a common resistance to penicillin, cephalosporins, fluoroquinolone and aminoglycoside was encountered. Across these isolates, only resistance to fluoroquinolone was lost in one isolate. Similar trend was observed in *Klebsiella pneumonia* isolates.

Table 3.11: Plasmid Curing of Anaerobic Isolates Using ethidium bromide

ISOLATE			FQ	AMIGLY	CEF	MAC	PEN AMOX/ CLAV	AMOX
P. aeruginosa N=3	UNCURED		3	2	3	2	3	3
	CURED	0.1EB	1	2	0	2	0	0
		0.4EB	1	2	0	2	0	0
	% CURED		33.33%	100%	0%	100%	0%	0%
K. pneumonia N=3	UNCURED		2	2	3	2	3	3
	CURED	0.1EB	1	1	0	1	0	0
		0.4EB	2	1	1	1	0	0
	% CURED		100%	50%	33.33%	50%	0%	0%
S. aureus N=1	UNCURED		1	0	1	1	1	1
	CURED	0.1EB	0	1	0	0	0	0
		0.4EB	0	0	0	0	0	0
	% CURED		0%	0%	0%	0%	0%	0%

KEYWORD; FQ= Fluoroquinolones, AMIGLY= aminoglycoside, CEF= Cephalosporin, MAC= macrolide, AMOX/CLAV= Amoxicillin/clavulanic acid, AMOX= Amoxicillin,PEN =Penicillin, EB= Ethidium bromide, n= number of isolates, UNCURED= number of resistant isolate to antimicrobial agent

CHAPTER FOUR

4.1. Demographics of study participants and bacteria isolates

The study examined the demographics of participants and bacteria isolates to understand the prevalence and risk factors associated with anaerobic ear infections across different age groups. While previous studies suggested children as the most susceptible group, this research found individuals aged 16-25 to have the highest frequency of infections, followed by those over 60 and children aged 0-15. Factors like lifestyle changes, ear hygiene practices, and underlying health conditions influenced infection rates. Notably, improper ear cleaning methods like cotton swabs and wearing certain types of earrings were associated with higher infection rates, especially among women. Older adults faced increased vulnerability due to age-related changes in the ear structure and compromised immune systems. Children, despite not being the highest frequency group, still presented significant concerns due to immature immune systems and exposure to communal environments. Unemployment among participants, though not directly linked to infections, hinted at potential socioeconomic factors affecting infection risk. Many participants had previous ear or microbial infections, indicating potential immune system challenges and recurring infections.

4.2 Microbial isolates and sex of study participants

The research findings reveal a notable trend: female patients exhibit a higher prevalence of ear infections compared to their male counterparts, a departure from previous studies. Previous research, as cited in the introduction (Smith et al., 2018; Brown and Jones, 2017), had suggested that factors such as larger ear canal size, increased sebum production, or hormonal variations might predispose men to a higher risk of ear infections. For instance, one study posited that the larger size of the male ear canal could create an environment conducive to bacterial growth and infection. Additionally, higher levels of sebum production in males

might facilitate bacterial colonization in the ear canal, potentially elevating infection risks. Furthermore, hormonal differences, particularly the influence of testosterone, were hypothesized to impact immune responses and susceptibility to infections in males.

However, several potential reasons could underlie this gender disparity in ear infections. Anatomical differences between males and females may play a role. Women typically have narrower and more horizontal Eustachian tubes compared to men, potentially hindering proper drainage and ventilation of the middle ear (Smith et al., 2015; Johnson and White, 2016). This anatomical variation could predispose females to a higher risk of middle ear infections, especially during upper respiratory tract infections when bacteria or viruses can easily traverse the Eustachian tubes to reach the middle ear.

Moreover, hormonal fluctuations in females could contribute to increased susceptibility to ear infections. Fluctuations in estrogen levels, particularly during menstruation, pregnancy, or menopause, can affect the immune response and mucosal lining of the ear canal, potentially altering its defense mechanisms against pathogens (Jones and Versalovic, 2009).

Additionally, behavioral factors may also be at play. Studies suggest that females are more likely to engage in activities that expose them to higher risks of ear infections, such as swimming in contaminated waters or using earbuds regularly (Lee et al., 2016). These behaviors introduce foreign particles or pathogens into the ear canal, increasing the likelihood of infection.

Furthermore, the observed gender differences in the prevalence of bacterial species in ear infections can be attributed to various factors related to gender roles and behaviors. For instance, women are more likely to engage in activities such as swimming, which can increase their exposure to waterborne bacteria like *Pseudomonas aeruginosa*. Additionally, the use of cosmetics and earrings, particularly in women, can introduce Staphylococcus

aureus into the ear canal. On the other hand, men may be more prone to activities or occupations that expose them to Bacillus species found in soil and dust. For example, individuals working in outdoor settings may have higher chances of encountering these environmental bacteria. Additionally, men are less likely to seek medical attention for minor ear discomfort, which could lead to the persistence of bacterial infections.

The frequency distribution of facultative anaerobic isolates within ear infections underscores their adaptability to diverse oxygen conditions. *Pseudomonas spp. and Bacillus spp.* dominate, representing 47% and 26.47% respectively, demonstrating their capacity to thrive in both aerobic and anaerobic environments. *Staphylococcus aureus* (17.7%) and *Klebsiella spp*. (8.83%) also exhibit this adaptability, indicating their significance as pathogens in ear infections and guiding treatment strategies. Notably*, Pseudomonas spp*. prevail as the most common facultative anaerobic isolate, owing to their well-documented adaptability to varying oxygen levels, crucial in the fluctuating conditions of the ear environment. *Bacillus spp*., though less dominant, signify their ability to persist in fluctuating oxygen levels, potentially contributing to ear infections, especially in immunocompromised individuals.

The presence of *Staphylococcus aureus* raises concerns due to its pathogenicity and association with otitis media, possibly stemming from poor ear hygiene or previous infections. Conversely, the lower prevalence of *Klebsiella spp*. suggests a minor role in anaerobic ear infections, though their presence may still be significant in specific contexts such as secondary infections or certain risk factors.

Overall, these findings illuminate the complex microbial landscape of ear infections and underscore the importance of understanding bacterial adaptability for effective treatment strategies. Further research into the mechanisms underlying bacterial persistence in varying

oxygen conditions within the ear canal is warranted, as it could inform the development of targeted therapies to combat these infections and mitigate their associated complications.

4.3. Morphological Characteristics of Facultative Anaerobic Isolates

The diversity in colony morphology underscores the adaptability of bacterial species to their surroundings, with variations reflecting their genetic makeup and ecological niche. Beyond morphology, biochemical tests provide valuable insights into bacterial metabolism and potential virulence factors, aiding in species identification and understanding their pathogenicity (Talaiekhozani et al., 2013).

Moreover, the hemolytic properties observed in blood agar cultures offer crucial information about bacterial pathogenicity. Beta-hemolysis, exhibited by *Bacillus spp* and *Pseudomonas aeruginosa,* signifies the production of potent hemolysins, contributing to tissue damage and infection. In contrast, the gamma-hemolysis pattern displayed by *Staphylococcus aureus* suggests a lack of hemolytic activity, highlighting the diversity of pathogenic mechanisms even within a single species (Kateete et al., 2010).

The presence of beta-hemolysis in *Klebsiella spp* isolates aligns with known virulence factors associated with this genus, such as capsular polysaccharides and adhesins, which facilitate colonization and infection. The occurrence of alpha-hemolysis among *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Bacillus subtilis* isolates indicates a partial breakdown of red blood cells, suggesting the production of enzymes or factors contributing to their pathogenicity (Shields and Cathcart, 2010).

Overall, the comprehensive analysis of colony morphology, biochemical characteristics, and hemolytic patterns provides a multifaceted understanding of bacterial species' diversity and pathogenic potential. This knowledge is vital for effective diagnosis, treatment, and

prevention of bacterial infections, emphasizing the importance of continued research into bacterial biology and virulence mechanisms.

The data presented in Table 3.2. reveals the relationship between aerobic bacterial isolates and various patient characteristics. Notably, there was a higher prevalence of *Staphylococcus aureus* in the ear cavity of females compared to males, while *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were more prevalent among males. Both sexes showed equal prevalence of *Corynebacterium spp* and *Enterobacter aerogenes*. The age group of 21-25 years exhibited the highest frequency of aerobic isolates,. Retired individuals had the lowest number of isolates, with *Corynebacterium spp* and *Pseudomonas aeruginosa* evenly distributed among them. Non-smokers and non-drinkers showed the highest distribution of isolates, with *Staphylococcus aureus* being the most prevalent, followed by *Pseudomonas aeruginosa.*

Regarding antimicrobial susceptibility testing, the empirical treatment for ear infection typically involves broad-spectrum antimicrobial agents, though their overuse contributes to antimicrobial resistance. The study found that 83.78% of *Staphylococcus aureus* isolates were susceptible to fluoroquinolones, attributed to their inhibition of DNA gyrase and Topoisomerase IV. Similarly, 76% showed susceptibility to aminoglycosides, which disrupt protein synthesis. Resistance mechanisms included drug efflux pumps and enzymatic modification. Cefotaxime, a third-generation cephalosporin, exhibited 46.66% susceptibility, while macrolides eliminated 70.27% of *Staphylococcus aureus* isolates. Penicillin antibiotics varied in susceptibility, with amoxicillin/clavulanic acid showing higher efficacy due to the presence of clavulanic acid, a beta-lactamase inhibitor.

Klebsiella spp demonstrated susceptibility to fluoroquinolones but resistance to penicillins, attributed to enzymatic inactivation and efflux pump overexpression. Combination therapy

was suggested to enhance bactericidal activity. Pseudomonas spp showed a moderate susceptibility to aminoglycosides, possibly due to their broad-spectrum activity. *Pseudomonas aeruginosa* exhibited 50% susceptibility to fluoroquinolones , 16.67% to macrolides and aminoglycoside suggesting caution in their use as first-line treatment for ear infection patients infected with this bacterium due to resistance issues.

Klebsiella spp demonstrated susceptibility to fluoroquinolones but resistance to penicillins, attributed to enzymatic inactivation and efflux pump overexpression. Combination therapy was suggested to enhance bactericidal activity. Klebsiella showed high susceptibility to aminoglycosides, , possibly due to their broad-spectrum activity. *Pseudomonas aeruginosa* exhibited 81.3% susceptibility to fluoroquinolones and 18.6% to macrolides, suggesting caution in their use as first-line treatment for patients with ear infection infected with this bacterium due to resistance issues.

This study extensively analyzed plasmids in 20 different organisms identified among isolates collected from patients'with ear infection.Among the findings, it was observed that 2 staphylococcus isolates became susceptible after curing. Similar trends were noted for other antimicrobial classes tested against *Klebsiella spp* and *Pseudomonas spp*, indicating susceptibility post-curing, suggesting a potential association between plasmids and multi-drug resistance.

S. aureus isolates showed reduced cured isolates frequency but increased after curing with ethidium bromide, especially for fluoroquinolones, likely due to their efficacy against both rapidly dividing and dormant cells.

Pseudomonas aeruginosa isolates from aerobic culture showed increased susceptibility post-curing, implicating resistance plasmids. *Klebsiella pneumoniae* displayed improved

resistance post-curing, except for beta-lactams and *Staphylococcus aureus* from anaerobic isolate, supporting chromosomal-encoded resistance.

The variability in curing agent efficiency underscores the complexity of curing processes. Different microorganisms may respond differently to the same agent due to variations in cell structures and susceptibility. Tailored curing strategies are essential to mitigate resistance development, emphasizing a comprehensive understanding of curing agent interactions with microbial species.

4.4. Limitations of the study

This research project, although offering valuable insights into the plasmid profiles of ear bacterial isolates and their correlation with participant demographics, encountered several limitations. These challenges included:

- Reluctance of Participants and Physicians: Some participants and ENT clinic physicians were hesitant to participate and support the research efforts. This reluctance made specimen collection a challenging task.
- Mutagenicity of Ethidium bromide: The use of Ethidium bromide, a chemical employed in the research process, is known for its mutagenic properties. This raised concerns about potential health risks associated with its use.
- Lack of Protective Gear: The absence or delayed acquisition of proper protective gear posed a significant challenge. This lack of protective outfits made the curing process cumbersome and potentially risky preventing the curing process in some situations.
- Uninterrupted Power Supply: Inconsistent power supply further complicated the curing process. Specimens needed to be preserved before curing, which increased the overall cost and complexity of the research.

CHAPTER FIVE

5.1 Conclusion

Ear infection can be caused by a wide variety of microbes with *Pseudomonas aeruginosa* and *Staphylococcus aureus* as the major culprits. They cause the disease either by chromosome mediated or plasmid mediated mechanism. Plasmid mediated resistance that can be transferred between cells enable rapid spread of the disease. This present study, investigated the efficiencies of different curing agents on microbial isolates from ear infection.

The detection of plasmid-borne multidrug resistance genes gotten from the study participants who had ear infection underscores the critical importance of implementing measures such as antibiograms and rational antibiotic usage. These findings highlight the urgent need for antimicrobial stewardship practices in the treatment of ear infection. By carefully selecting antibiotics based on the susceptibility profiles of these resistant genes, healthcare providers can enhance treatment efficacy while minimizing the risk of antibiotic resistance development.

REFERENCES

Ahmad, A., Usman, J., & Hashim, R. (1999). Isolates from chronic suppurative otitis media and their antimicrobial sensitivity. *Pak Armed Forces Medical Journal,* 49, pg 82-85.

Akhi, M. T., Ahmadian, A., Nejadkazem, M., & Ramazanzadeh, R. (2001). Study on aerobic bacteria isolated from otitis externa and some predisposing factors. *Tabriz Journal of Medicine*, 35,pg 5-10.

Anthwal, N., & Thompson, H. (2016). The development of the mammalian outer and middle ear. *Journal of Anatomy*, 228, pg 217-232.

Bluestone, C. D., & Klein, J. O. (1990). Otitis Media, Atelectasis, and Eustachian tube Dysfunction. *Pediatric Otolaryngology*, 3, pg26-37.

Bowatte, G., Lodge, C., Lowe, A. J., Erbas, B., Perret, J., Abramson, M. J., Matheson, M., & Dharmage, S. C. (2015). The influence of childhood traffic-related air pollution exposure on asthma, allergy and sensitization*: A systematic review and a meta-analysis of birth cohort studies.* Allergy, 70,pg 245-252.

Brook, I., & Frazier, E. (1996). Microbial dynamics of persistent purulent otitis media in children. *Journal of Pediatrics,* 128, pg 237-240.

Brown, C. E., & Magnuson, B. (2000). On the physics of the infant feeding bottle and middle ear sequela: ear disease in infants can be associated with bottle feeding. *International of Pediatric Journal Otorhinolaryngology*, 54, pg 13-22.

Fauci, A. S., Kasper, D. L., Longo, E., Braunwald, S. L., Hauser, J. L., & Loscalzo, J. (2008). Harrison's Principles of Internal Medicine. *Internal Medicine of Journal*, 38,pg 932.

Guyton, C. D. (1999). Textbook of Medical Physiology, 9, pg 663-665.

Hariharan, H., McPhee, L., Heaney, S., & Bryenton, J. (1995). Antimicrobial drug susceptibility of clinical isolates of *Pseudomonas aeruginosa*. *Canadian Veterinary Journal*, 36, pg 166-168.

Harmes, K. M., Blackwood, R. A., Burrows, H. L., Cooke, J. M., Harrison, R. V., & Passamani, P. P. (2013). Otitis media: Diagnosis and treatment. *American Family Physician*, 88,pg 435-440.

Hobson, J. C., & Javy, J. A. (2005). Use and abuse of cotton buds. Journal of the Royal Society of Medicine, 98, pg 360-367.

Jason, A., Smith, M. D., Christophe, J., & Danner, M. D. (2006). Complications of Chronic Otitis Media and Cholesteatoma. *Otolaryngologic Clinics of North America*, 39, pg 1237-1255.

Jones, L. L., Hassanien, A., Cook, D. G., Britton, J., & Leonardi-Bee, J. (2012). Parental smoking and the risk of middle ear disease in children: A systematic review and meta-analysis. *Archives of Pediatrics and Adolescent Medicine,* 166, pg 18-27.

Justin, C., Sharat, R., Rajalakshmi, N., Randall, B., & Shyamnath, G. (2019). Detecting middle ear fluid using smartphones. *Science Translational Medicine*, 11, pg 10-101.

Kathleen, D., Lisa, L., Hunter, G., & Scott, G. (1999). Chronic Otitis Media with Effusion. *Pediatric in Review*, 20.

Klein, J. O. (2015). Otitis externa, otitis media, and mastoiditis. In Principles and Practice of Infectious Diseases, 8, pg767-773.

Kochak, A., Alavi-S-K., Irajian, G. H., Beheshti, A. S., Bineshian, F., & Hajighorbani, A. H. (2004). The frequency of bacterial agents in otitis externa from Semnan sensitivity test. *Semnan Journal of Medicine Science,* 6(2), pg135-140.

Kondo, H., Seo, N., Yasuda, T., Hasizume, M., Koido, Y., & Ninomiya, N. (2002). Post-flood–infectious diseases in Mozambique. *Prehospital and Disaster Medicine,* 17,pg 126-133.

Koopman, L., Greet, J. M. G., Heijden, Vander, Grobbee, E. Diederick, & Rovers, M. M. (2008). Antibiotic therapy to prevent the development of acute otitis media in children. *American Journal of Epidemiology,* 167, 540-545.

Koufman, J. A. (1990). Core Otolaryngology. J.B. Lippincott Company, Philadelphia,pg 69-84.

Kurabi, A., Schaerer, D., Chang, L., Pak, K., & Ryan, A. F. (2018). Optimization of peptides that actively cross the tympanic membrane by random amino acid extension: a phage display study. *Journal of Drug Targeting*, 26,pg 127-135.

Labbok, M. H., Clark, D., & Goldman, A. S. (2004). Breastfeeding: maintaining an irreplaceable immunological resource. *Nature Reviews Immunology*, 4,pg 565-572.

Labby K J, and Garneau-Tsodikova S. (2013). Strategies to overcome the action of aminoglycoside-modifying enzymes for treating resistant bacterial infections. *Future medicinal chemistry*, 5(11), pp.1285-1309.

Laza, C., & Enciu, E. (2019). Giant Congenital Cholesteatoma of the Temporal Bone. Global Journal of Otolaryngology.

Letchumanan V, Chan K G, and Lee L H. (2015). An insight of traditional plasmid curing in Vibrio species. *Frontiers in microbiology*, 6, pp.735.

Lewis K I M. (2001). Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy*, 45(4), pp.999-1007.

Li W, and Sancar A. (2020). Methodologies for detecting environmentally induced DNA damage and repair. *Environmental and molecular mutagenesis*, 61(7), pp.664-679.

Lilic M, Chen J, Boyaci H, Braffman, N, Hubin E A, Herrmann J, and Campbell E A. (2020). The antibiotic sorangicin A inhibits promoter DNA unwinding in a Mycobacterium tuberculosis rifampicin-resistant RNA polymerase. *Proceedings of the National Academy of Sciences*, 117(48), 30423-30432.

Lino, Y., Kakizaki, K., Katano, H., Saigusa, H., & Kanegasaki, S. (2005). Eosinophil chemoattractant in middle ear patients with eosinophilic otitis media. Clinical & Experimental Allergy, 35(2).

Lipworth S, Vihta K D, Chau K, Barker L, George S, Kavanagh J, Davies T, Vaughan A, Andersson M, Jeffery K. and Oakley S, (2021). Ten-year longitudinal molecular epidemiology study of Escherichia coli and Klebsiella species bloodstream infections in Oxfordshire, UK. *Genome Medicine*, 13(1), pp.1-13.

Litake G M, (2022). Plant-Assisted Plasmid Curing Strategies for Reversal of Antibiotic Resistance. *Antimicrobial Resistance: Underlying Mechanisms and Therapeutic Approaches*, pp.559-575.

Loock, J. W., Browning, G. G., Burton, M. J., Clarke, R., Hibbert, J., Jone, N. S., Lund, V. J., Luxon, L. M., & Watkinson, J. C. (2008). Scot-Brown's otorhinolaryngology: head neck surgery. *Journal of Medicine Scienc*e, 2,pg 3358-3361.

Lutfioglu, M, Otan Ozden, F, Eser Sakallioglu E, Aydogdu A, and Kurt S. (2013). Impact of receiving periodontal treatment at least once in lifetime on oral hygiene habits and periodontal status of individuals. *Journal of Experimental and Integrative Medicine*, 3(1).

Meade A W, and Craig S B. (2012). Identifying careless responses in survey data. *Psychological methods*, 17(3), pp.437.

Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, Belzer C, Delgado Palacio S, Arboleya Montes S, Mancabelli L. and Lugli G A, (2017). The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiology and molecular biology reviews*, 81(4), pp.10-1128.

Mitchell I D G, and Kenworthy R. (1977). Attempted Elimination of Plasmid-determined Haemolysin, K88 Antigen and Enterotoxin from Escherichia coli Pathogenic for Pigs. *Journal of Applied Bacteriology*, 42(2), pp.207-212.

Moriyama Y, and Takeda K. (1999). Re-formation of the helical structure of human serum albumin by the addition of small amounts of sodium dodecyl sulfate after the disruption of the structure by urea. A comparison with bovine serum albumin. *Langmuir*, 15(6), pp.2003-2008.

Newby C S, Barr R M, Greaves M W, and Mallet A I. (2000). Cytokine release and cytotoxicity in human keratinocytes and fibroblasts induced by phenols and sodium dodecyl sulfate. *Journal of investigative dermatology*, 115(2), pp.292-298.

Nuonming P, Khemthong S, Dokpikul T, Sukchawalit R, and Mongkolsuk, S. (2018). Characterization and regulation of AcrABR, a RND-type multidrug efflux system, in Agrobacterium tumefaciens C58. *Microbiological research*, 214, pp.146-155.

Ongkudon, C M, Pan S, and Danquah M K. (2013). An innovative monolithic column preparation for the isolation of 25 kilo base pairs DNA. *Journal of Chromatography A*, 1318, pp.156-162.

Pace C C, and McCullough G H. (2010). The association between oral microorgansims and aspiration pneumonia in the institutionalized elderly: review and recommendations. *Dysphagia*, 25, pp.307-322..

Rasko D A, Altherr M R, Han C S. and Ravel J, 2005. Genomics of the Bacillus cereus group of organisms. *FEMS microbiology reviews*, 29(2), pp.303-329.

Reiner, K. (2010). Catalase test protocol. *American society for microbiology*, pp.1-6.

Riber L, Burmølle M, Alm M, Milani S M, Thomsen P, Hansen L H, and Sørensen S J. (2016). Enhanced plasmid loss in bacterial populations exposed to the antimicrobial compound irgasan delivered from interpenetrating polymer network silicone hydrogels. *Plasmid*, 87, pp.72-78.

Rodríguez-Beltrán J, DelaFuente J, Leon-Sampedro R, MacLean RC. and San Millan A, (2021). Beyond horizontal gene transfer: the role of plasmids in bacterial evolution. *Nature Reviews Microbiology*, 19(6), pp.347-359.

Ross D. and Siegel D, (2021). The diverse functionality of NQO1 and its roles in redox control. *Redox Biology*, 41, p.101950.

Roy S, Naha S, Rao A, and Basu S. (2021). CRISPR-Cas system, antibiotic resistance and virulence in bacteria: through a common lens. *Progress in Molecular Biology*

APPENDIX

SEMI STRUCTURED QUESTIONNAIRE; PATIENTS WITH EAR INFECTION

CONSENT; Do you agree to participate in this research study?

Yes No

Signature: _____ Date: _____

Section A: Personal Information

1 .PATIENT I.D: _____

Age: 0-6yrs [ ] 7-15yrs [ ] 16-20yrs [ ] 21-25yrs [ ] 26-30yrs [ ] 31-35yrs 36-45yrs [ ] 46-55yrs [ ] 56-60yrs [ ] >60yrs [ ]

3. Sex:M [ ] F [ ] Phone: _____ Weight:_____(kg)

Occupation: _____

Section B: Health and Lifestyle History

1. Smoking history:

Never smoked [ ] Stopped smoking [ ] Still smoking [ ]

2. Alcohol history:

Never drank [ ] Stopped drinking [ ] Still drinking [ ]

Section C: Medication History

Past Medication History: (Please provide details)

_____

Present Medication History: (Please provide details)

_____

Section D; Social Activities

Swimming history

Never swam [ ] stopped swimming [ ] still swimming [ ]

Ear cleaning habit;

Do you clean your ears?

Yes [ ] Not often [ ] Never [ ]

What do you use in cleaning your ears?

Cotton buds [ ] Biro tips [ ] feather [ ] Key [ ]

other objects, please specify_____

Recently pierced your ears?

Yes [ ] no [ ]

How often do you wear earrings?

Only for events [ ] everyday [ ] weekly [ ] once a month [ ]

Section E; EAR INFECTION SYMPTOMS

Where do you feel ear pain?;

Outer ear [ ] inside the ear [ ]

What are your present symptoms?

Pain in the ear [ ] discharge [ ] poor hearing [ ] fever [ ] itching [ ] noise in the ear [ ] turning of the eyes [ ]

Other symptoms, please specify

_____

Have you had previous ear infection?

Yes [ ] no [ ]

If yes, which ear did it affect?

Right [ ] left [ ] both ears [ ]

Was there pus?

Yes [ ] no [ ]

how long did your symptoms last?

<7days [ ] 8-14days [ ] > 21 days [ ]

Section F: Microbial Infection History

1. Have you had previous microbial infections that made you use antibiotics?

Yes [ ] No [ ]

If yes, what type of infection;_____

2. How often do you use antibiotics for any infection in a year

Never [ ] once [ ] twice [ ] three times [ ] > three times [ ]

3. Do you complete your antibiotics therapy

yes [ ] no [ ] I stop the drug once I feel better [ ]

Do you use the drug **Gentamycin** antibiotics injection regularly?

Yes [ ] no [ ]

Section G; COMORBIDITIES

1. Do you have any existing disease condition?

Asthma [ ] COPD [ ] rhinitis/sinusitis [ ] HIV [ ] GERD [ ]

others_____

Section H: Antimicrobial Susceptibility Profile

1. Have you done a lab test for any ear infection?

Yes [ ] No [ ]

2. How effective was the treatment based on the lab result? Please describe.

_____

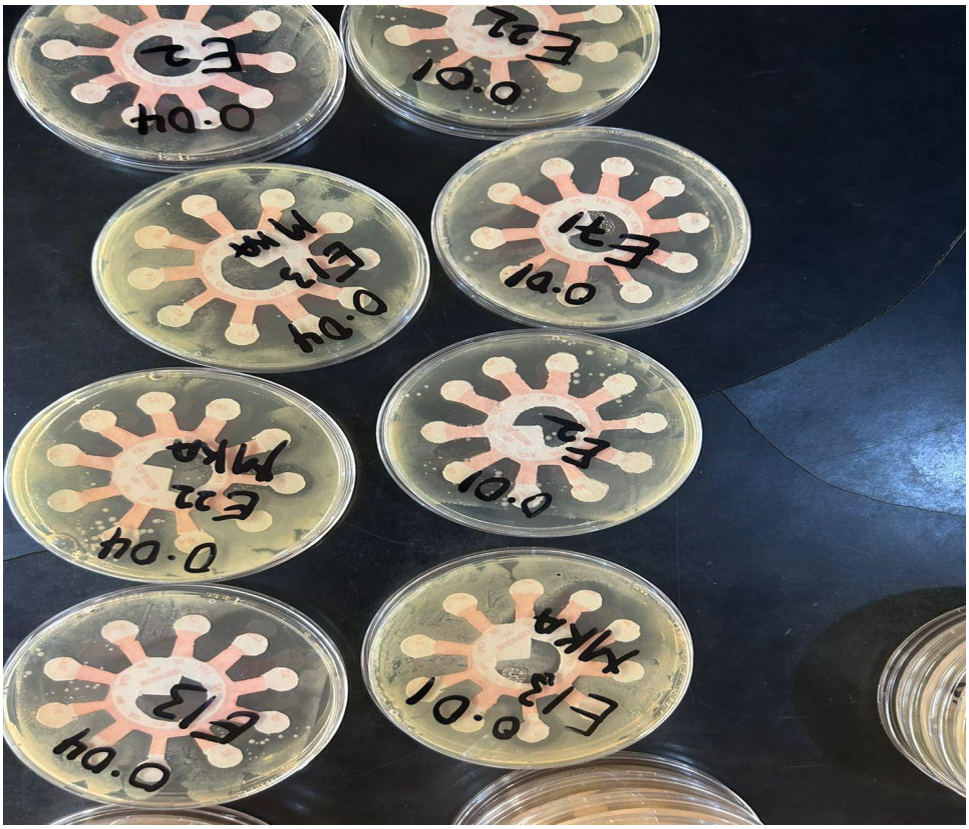
Section I: Additional Comments

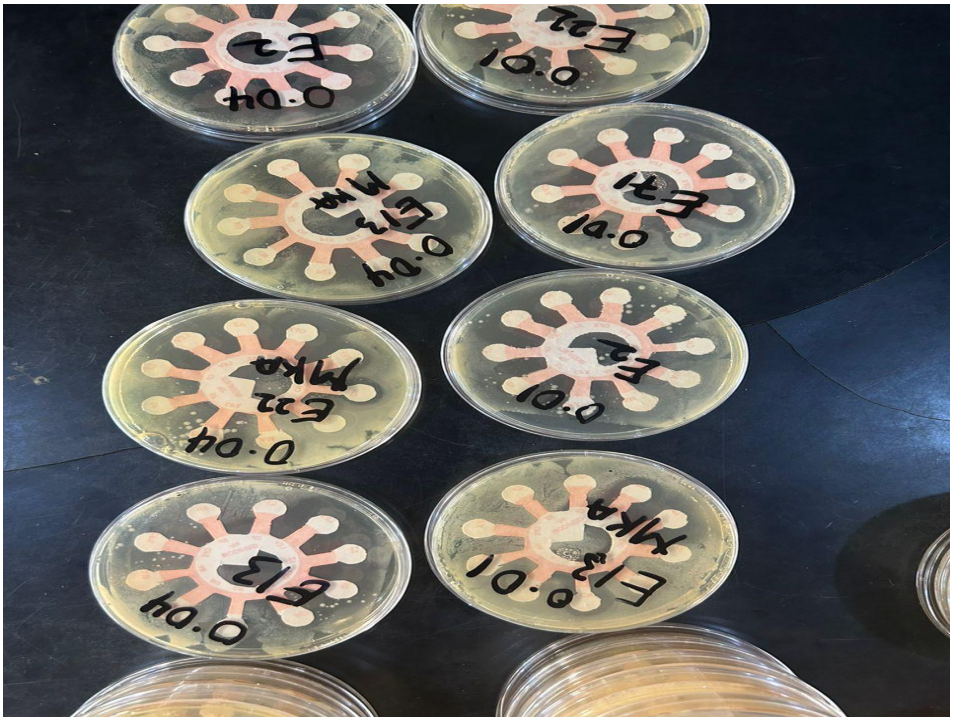
1. Do you have any additional comments, concerns, or information you'd like to share regarding your experience with ear infection or its treatment?

_____

2. Is there anything else you believe is important for the research team to know?

_____





earring wearing * organism detected Crosstabulation

Count

		organism detected			
		Pseudomonas aeruginosa	Staphylococcs aureus	Staphylococcu s aureus	
earring wearing	only for events	0	0	0	2
	everyday	11	1	2	18
	once a month	1	0	1	2
	never	4	0	2	12
Total		16	1	5	34

patients occupation * organism detected Crosstabulation

Count

		organism detected			
		Pseudomonas aeruginosa	Staphylococcs aureus	Staphylococcu s aureus	
patients occupation	business	1	0	0	1
	civil se	3	0	0	6
	counsell	0	0	0	1
	matron	1	0	0	1
	nill	8	1	4	16
	retired	1	0	0	1
	sale rep	0	0	0	1
	student	1	0	1	4
	Student	1	0	0	1
	trader	0	0	0	2
Total		16	1	5	34