Biochemical characterization and antibiotic resistance of some medically important bacterial isolates

A Dissertation
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By
Prabhu Prasad Sarangi
(409LS2056)

Under the guidance of
Dr Surajit Das
Assistant Professor
Department of Life Science

Department of Life science
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008(ORISSA)

NATIONAL INSTITUTE OF TECHNOLOGY
Department of Life science
Rourkela-769008, Orissa, india

Dr. Surajit Das, Ph.D
Asst. Professor

CERTIFICATE

This is to certify that the dissertation entitled "**Biochemical characterization and antibiotic resistance of some medically important bacterial isolates**" Submitted by Mr. Prabhu Prasad Sarangi, to the department of Life science, National Institute of Technology, Rourkela for the degree of Master of Science in Life science is based on the result obtains in the bonafide project work carried out by him under my Guidance and supervision.

I further certify that to the best of my knowledge Prabhu Prasad Sarangi bears a good moral character.

Dr. Surajit Das
(Asst. Professor)

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In the end I must record my special appreciation to GOD who has always been a source of my strength, inspiration & my achievements.

PRABHU PRASAD SARANGI

DECLARATION OF CANDIDATE

I hereby declare that the research work incorporated in this dissertation entitled **"Biochemical characterization and antibiotic resistance of some medically important bacterial isolates"** is an authentic research work carried at Department of Life science, National Institute Technology, Rourkela under the direct guidance and supervision of Dr. Surajit Das, Asst. Professor, Department of Life science, NIT, Rourkela.

Date: **Mr. Prabhu Prasad Sarangi**

CONTENTS

LIST OF TABLES

LIST OF PHOTOGRAPHS

LIST OF SYMBOLS AND ABBREVIATIONS

MIC	Minimum Inhibitory Concentration
UTIs	Urinary Tract Infections
CDC	Center for Disease Control
CFU	Colony Forming Unit
MDRPA	Multi-Drug Resistant *Pseudomonas aeruginosa*
SIRS	Systemic Inflammatory Response Syndrome
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
MR-VP	Methyl Red-Voges proaskar
µg	Microgram
mL	Milliliter
mg	Milligram
T	Tetracycline
V	Vancomycin
Am	Amoxicillin
Cf	Ciprofloxacin
K	Kanamycin
C	Chloramphenicol
pH	H^+ ion concentration

ABSTRACT

Pathogens are treated with antibiotics from years, bacterial pathogens are either resistant or sensitive to antibiotics. Regular administration of antibiotic to pathogen produces drug resistance strains. Most of the bacterial pathogen already acquires multi-drug resistance characteristics. *Pseudomonas aeruginosa* is resistance to almost all antibiotics for example; tetracycline,kanamycin, chloramphenicol , vancomycin etc..the MIC study of these antibiotics shows the concentration at which these pathogens can be inhibited. For *pseudomonas*, vancomycin MIC is 32 µg/ml, penicillin is 8 µg/ml, chloramphenicol 16 µg/ml. so also for *proteus mirabilis* vancomycin 16 µg/ml penicillin 4 µg/ml

INTRODUCTION:-

A pathogen is a microorganism such as a virus, bacterium, prion or fungus that causes disease in its animal or plant host. Pathogenic bacteria causes bacterial infection. Some species of bacteria, such as *Burkholderia cenocepacia*, *Pseudomonas aeruginosa*, and *Mycobacterium avium*, are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression or cystic fibrosis. (Heise, 1982; Saiman, 2004).

Potency:

A hypothesis regarding pathogens states, the longer a pathogen can survive outside of the body, the more dangerous it is to a potential host. For example, the pox virus (*Variola virus*) also survive outside the host for 885 days. It is one of the deadliest pathogenic viruses, responsible for 20-50% of deaths by infectious. The tuberculosis bacterium kills 1 of 5 people it infects, but has a life of only 244 days outside the host. However, research on the ability of pathogens to cause disease provides evidence from multiple and diverse species of the existence of pathogenicity or virulence factors, encoded within the pathogens' genetic material, that facilitate microbes to cause disease. In countries that having higher sanitation standards, pathogens cannot survive for as long outside of the human. This leads to increased rate of mutation in the pathogen which would make it less deadly. These mutations allow the pathogen to survive in the host for longer periods of time.

Treatment

Antibiotics used to combat bacterial infections can be classified as bactericidal which kill bacteria, or bacteriostatic which prevent bacterial growth. Different antibiotics inhibit distinct process in the pathogen which are different from that found in the host. For example, the antibiotics chloramphenicol and tetracycline inhibit the bacterial ribosome, but not the structurally-different eukaryotic ribosome, and so exhibit selective toxicity (Yonath and Bashan, 2004). Other than treating human diseases, antibiotics are extensively used in farming practices for preventing crop infections and both these processes aid in rapid development of antibiotic resistance in bacterial populations. (Khachatourians, 1998). Infections can be minimized

by antiseptic measures such as sterilizing the skin before to piercing it with the needle and by proper care of indwelling catheters. Dental and surgical instruments are also sterilized to prevent infection by bacteria. Disinfectants are used to kill bacteria and other pathogens on surfaces to prevent contamination and further reduce the risk of infection. Most bacteria in food are sterilized by cooking to temperatures above 73 °C (163°F).

SCIENTIFIC CLASSIFICATION:-

Proteus mirabilis:	Kingdom: Bacteria
	Phylum: Proteobacteria
	Class : Gamma proteobacteria
	Order: Enterobacteriales
	Family: Enterobacteriaceae
	Genus: *Proteus*
	Species: *P. mirabilis*

Pseudomonas aeruginosa:	Kingdom: Bacteria
	Phylum: Proteobacteria
	Class: Gammaproteobacteria
	Order: Pseudomonadales
	Family: Pseudomonadaceae
	Genus: *Pseudomonas*
	Species: *P. aeruginosa*

Pseudomonas

Pathogenic members of this genera include *P. aeruginosa*, *P. oryzihabitans*, and *P. plecoglossicida*. *P. aeruginosa* founds in hospital environments and is a common problem in this environment since it is the second most common causative agent of nosocomial infections. This pathogenesis may in part be due to the metabolites secreted by *P. aeruginosa*. The bacterium possesses a wide range of secretion systems, which secrets numerous proteins relevant to the pathogenesis of clinical strains (Hardie et al. 2009). Like most bacteria genera the *pseudomonas* last lived hundreds of million years ago; although they were classified by humans at the end of the XIX century. Due to their widespread occurrence in water and in plant seeds such as dicots, the pseudomonads were observed early in the history of microbiology. The genus *Pseudomonas* created for these organisms was defined in rather vague terms by W. Migula in 1894 and 1900 as a genus of rod-shaped, Gram-negative and polar-flagella bacteria with some sporulating species, the statement was later proved incorrect and due to refractive granules of reserve materials. Despite the vague description, the type species, *Pseudomonas aeruginosa* proved the best descriptor. Additionally, the etymology of the name was not provided and first appeared in the Bergey's manual (=top authority in bacterial nomenclature) as Greek pseudes (false) and monas (a single unit), which can mean false unit, but there is also the possibility that Migula intended it as false Monas, a non flagellate protist. Subsequently, the term "monad" was used in the early history of microbiology to denote single-celled organisms. Soon afterwards, *pseudomonas* were isolated from many natural niches, and a large number of species names were originally assigned to the genus. New methods and the extent of approaches based on the studies of conservative macromolecules have reclassified many strains. *Pseudomonas aeruginosa* is a common bacterium which is able to cause disease in animals, including humans. It is found in water, soil, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or people with reduced immunity. The symptoms of such infections are generalized inflammation and septic. If such infection occurs in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal (Balcht et al, 1994). Because it thrives on most surfaces, this bacterium is also found on and

in medical equipment, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills (Balcht et al, 1994; Itah and Essien, 2005).

IDENTIFICATION

It is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility (Ryan and Ray, 2004) An opportunistic human pathogen, *P. aeruginosa* is also an opportunistic pathogen of plants (Iglewski, 1996). *P. aeruginosa* is the type speciesof the genus *Pseudomonas* (Migula) (Anzai *et al, 2000). P. aeruginosa* secretes a variety of pigments, including pyoverdine (yellow-green and fluorescent), pyocyanin (blue-green) and pyorubin (red-brown). King, Ward, and Raney developed Pseudomonas Agar P (King A medium) for enhancing pyocyanin and pyorubin production, and Pseudomonas Agar F (King B medium) for enhancing fluorescein production (King et al, 1954). *P. aeruginosa* is often preliminarily identified by its pearl like appearance and grape-like or tortilla-like odor *in vitro*. Definitive clinical identification of *P. aeruginosa* moreover includes identifying the production of pyocyanin and fluorescein, as well as its ability to grow at 42°C. *P. aeruginosa* is capable of growth in diesel and jet fuel, where it is known as a hydrocarbon-using microorganism, causing microbial corrosion. It creates dark, gellish mats sometimes improperly called "algae" due to their appearance. Although classified as an aerobic organism, *P. aeruginosa* is considered by many as a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate as aterminal electron acceptor, and, in its absence, it is also able to ferment arginine by substrate-level phosphorylation (Palmer et al, 2007; Vander et al, 1984). Adaptation to anaerobic or microaerobic environments is necessary for certain lifestyles of *P. aeruginosa*, for example, during lung infection in cystic fibrosis patients, where thick layers of lung mucus and alginate surrounding mucoid bacterial cells can limit the diffusion of oxygen (Hassett, 1996; Worlitzsch et al, 2002; Williams et al, 2007; Brown, 1956).

Proteus:

P. vulgaris, *P. mirabilis* and *P. penneri*are opportunistic human pathogens. *Proteus* includes pathogens for many human urinary tract infections. (Guentzel, 1996). *P. mirabilis* causes wound and urinary tract infections. Most strains of *P. mirabilis* are sensitive to ampicillin and cephalosporins. *P. vulgaris* is not sensitive to these antibiotics. However, this organism is isolated least in the laboratory and usually only targets immunosuppressed individuals. *P. vulgaris*occurs naturally in the intestines of humans and a wide variety of animals; also manure, soil and polluted waters. *P. mirabilis*, once attached to urinary tract it infects the kidney more commonly than *E. coli*. *P. mirabili*s are often found as free-living organisms in water and soil. *Proteus* species do not usually ferment lactose, but have shown to be capable lactose fermenters depending on the species in a triple sugar iron (TSI) test. Since it belongs to the family of *Enterobacteriaceae*, general behaviors are applied on this genus: It isoxidase-negative, but catalase- and nitrase- positive. Specific tests includes positive urease (which is the fundamental test to differentiate *Proteus* from *Salmonella*) and phenylalanine deaminase tests. On the species level, indole is considered reliable, because it is positive for *Proteus vulgaris* but negative for *Proteus mirabilis*.

OBJECTIVES:-

1. Isolation and pure culture of *Proteus* and *Pseudomonas*

2. Biochemical characterization of pathogens.

3. Resistance properties of *Proteus* and *Pseudomonas* toward various antibiotics.

4. Determination of Minimum Inhibitory Concentration (MIC) of some important antibiotics toward *Proteus* and *Pseudomonas*

3.1. Pseudomonas

3.1.1. Disease

Pathogenic microbes are widespread in nature, inhabiting soil, water, plants, and animals (including humans). *Pseudomonas aeruginosa* has become an important agent of infection, especially in patients with compromised host defense mechanisms. It is the most common pathogen extracted from patients who have been hospitalized longer than 1 week. It is a frequent cause of nosocomial infections such as urinary tract infections (UTIs), pneumonia, and bacteremia. Pseudomonal infections are risky and can be life threatening. *Pseudomonas aeruginosa* is the opportunistic pathogen of humans. It is a major cause of hospital-acquired infections (nosocomial infections), and it is difficult to eradicate due to its resistance to most antimicrobial agents. There is probably no tissue that cannot become infected by *Pseudomonas* if the host defenses are weakened, and it is difficult to treat due to inherent and acquired resistance to antimicrobial agents. It is usually involved in urinary tract infections, soft tissue infections and pneumonia

3.1.2. Pathophisiology

P. aeruginosa is an opportunistic pathogen. It rarely causes disease in healthy persons. In most cases of infection, the integrity of a physical barrier to infection (eg. mucous membrane, skin) is lost or an underlying immune deficiency (eg, neutropenia, immunosuppression) is present. In addition to its pathogenicity, this bacterium has minimal nutritional requirements and can tolerate a wide variety of physical conditions. The pathogenesis of pseudomonas infections is multifactorial and complex. *Pseudomonas* species are both invasive and toxigenic. The 3 stages, are (1) bacterial attachment and colonization, (2) local infection, and (3) bloodstream dissemination and systemic disease. (Pollack, 2000). The importance of adherence and colonization is most evident when studied in the context of respiratory tract infection in patients with cystic fibrosis and in those that complicate mechanical ventilation. Production of extracellular proteases increases the organism's virulence by assisting in bacterial adherence and invasion. As per the Centers for Disease Control and Prevention (CDC), the overall prevalence of *P aeruginosa* infections in US hospitals is approximately 4 per 1000 discharges (0.4%).*P*

aeruginosa is also one of the most commonly isolated nosocomial pathogen, accounting for 10.1% of all hospital-acquired infections. It is found on the skin of some healthy persons and has been isolated from the throat and stool of 5% and 3% of nonhospitalized patients, respectively. The gastrointestinal carriage rates among hospitalized patients increases to 20% within 72 hours of admission.

Inhibits proliferation of human bone marrow progenitor cells:

Pseudomonas aeruginosa exotoxin A, it is a potent inhibitor of eukaryotic protein synthesis, is produced in vivo at the time of human infection. We tested that the hypothesis that exotoxin A may be responsible for the leukopenia which sometimes accompanies pseudomonas disease by examining the in vitro toxicity of exotoxin A for human bone marrow granulocyte-macrophage progenitor cells (colony-forming units in culture [CFU-c] in the soft agar cloning system. Colonization by freshly obtained marrow cells from five normal subjects was inhibited by exotoxin A in a concentration-dependent manner. The mean 50 and 100% inhibitory concentrations of toxin were 1.4 x 10(-10) and 1.4 x 10(-8) M, respectively, also significant inhibition was observed at a toxin concentration as low as 1.4 x 10(-13) M in two subjects. The inhibitory effect of exotoxin A on colony growth was specifically neutralized by antiserum to exotoxin A. Although mouse CFU-c were somewhat less sensitive to exotoxin A in vitro compared with human CFU-c, exotoxin A produced significant leukopenia in vivo in mice. These data gives a possible mechanism for the leukopenia which sometimes occurs in human pseudomonas disease. (Stuart and Pollack, 1982). Infectious morbidity may occur after endoscopy despite negative surveillance cultures. The process of routine endoscope cultures does not prevent device-linked infectious morbidity. (Fraser et al, 2004).

3.1.3. Clinical Feature

Clinical samples yield one or another of two smooth colony types. One type has a fried-egg appearance and is large, with flat edges, smooth and an elevated appearance. Another type, frequently found from respiratory and urinary tract secretions, has a mucoid appearance, is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play a key role in colonization and virulence. *P. aeruginosa* produce two types of soluble

pigments,the blue pigment pyocyanin and the fluorescent pigment pyoverdin. The former is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") known as "blue pus", which is a characteristic of infections caused by *Pseudomonas aeruginosa*.

3.1.4. PATHOGENESIS

Outside the hospital most common infection caused by *P.aeruginosa* is suppurative otitis, which is chronic though not disabling. In the hospital, it may cause localised or generalised infections. Localized lesions are commonly infections of wounds, eye infections and urinary infections folowing catheterisation. *P. aeruginosa* is the most common and serious cause of infections in burns. It is also one of the agent s responsible for iatrogenic meningitis following lumbar puncture. It frequently causes post-tracheostomy pulmonary infection. Septicemia and endocarditis may occur in patients who are debilitated due to concomitant infection. Malignancy or immunosuppressive therapy. Ecthyma gangrenosum and many other types of skin lesions have been described occuring either alone or as part of generalised infection, mainly in patients with leukemia and other types of malignancy. Infection of the nail bed is not uncommon following axcessive exposure of hands to detergents and water. *P. aeruginosa* has been described as one of the agents responsible for infantile diarrhea and sepsis. Strains isolated from outbreaks of diarrhea may from a heat liable enterotoxin nad give a positive rabbit ileal loop reaction. *P. aeruginosa* has been reported to cause a self-limited febrile illness (Shanghai fever) resembling typhoid fever in some tropical areas. The pre-eminent role of *P.aeruginosa* in hospital infection is due to its resistance to common antibiotics and antiseptics, and its ability to establish itself widely in hospitals. Being an extremely adaptable organism it can survive and multiply even with minimal nutrients, if moisture is available. Equipment such as respirators and endoscopes, articles such as bed pans and medicines such as lotions, ointments and eye drops and even stocks of distilled water or plants and flowers may be frequently contaminated. *P. aeruginosa* is present on the skin of the axial and perineum in some persons. Fecal carriage is not common but may be frequent following oral antibiotics treatment or hospitalization. The mechanism of pathogenesis

is not clearly understood. Several toxic extracellular products have been identified in the culture filtrates, such as exotoxins A and S. exotoxins 'A' acts as NADase, resembling the diarrheas toxin. Good antibody response to exotoxin A is considered a favourable sign in severe infections with *P. aeruginosa*. Other toxic products include protease, elastases, hemolysins and enterotoxins. The slime layer acts as a capsule in increasing virulence.

3.1.5. Frequency of multidrug-resistance *pseudomonas aeruginosa:*

The frequency of MDRPA infections is difficult to ascertain. Survival study evaluating in vitro susceptibility of commonly used antibiotics against clinical isolates of *P. aeruginosa* provides estimations of the rate of MDRPA infections. (Karlowsky et.al, 2003) However, there are a few limitations to consider. First, most of these surveillance studies did not use molecular techniques to eliminate clonal spread; thus, results may be an overestimation in outbreak situations. Second, these studies do not separates clinical infection from colonization. Finally, studies fails in standardization in definitions of multi drug resistance, which impedes direct comparisons of rates among studies. Because of these limitations, the trends in the isolation of MDRPA should be emphasized rather than actual rates reported. Studies meeting our definition of multi drug resistance reported increasing trends in MDRPA. From 1997-1999, the SENTRY Antimicrobial Surveillance Program published MDRPA rates of 8.2% in Latin America, 4.7% in Europe, 1.6% in Asia-Pacific, 1.2% in the United States, and 0.9% in Canada.(Gales, et al 2001). Among 1215 isolates of *P. aeruginosa* collected from 1999-2000 in Japan, 3% were found to be multidrug-resistant. (Kitahashi et al, 2001)**.**The two recent surveillance studies reported increases in MDRPA in the United States. (Karlowsky et al, 2003) (Obritsch et al, 2004). From 1998-2001, the Surveillance Network noted that multidrug resistance increased from 5.5% to 7.0% of *P. aeruginosa* isolates in patients not in an intensive care unit and from 7.4% to 9.1% in patients in an intensive care unit. (Karlowsky et al, 2003). The Care Unit Surveillance Study reported a significant increase in MDRPA isolates from 4% in 1993 to 14% in 2002 ($p<0.0001$) (Obritsch et al, 2004). Rates of MDRPA in individual institutions are even higher than those reported in large surveillance studies. In a university hospital in Brazil, 10.2% of *P. aeruginosa* isolates (48/472) were reported as MDRPA in 1992 (Arruda et al, 1999). An institution in Greece indicated that 24.3% (25/103) of non-duplicate *P. aeruginosa* isolates from 1996-1997 were

resistant to almost all antimicrobials tested on it. (Sofianou and Tsakris, 1997) Among resistant strains of *P. aeruginosa* isolated from 1994-1998 in a French hospital, 24% (8/34) were reported as MDRPA. (Trouillet and Vuagnat, 2002). A university hospital in Italy reported a single case of MDRPA isolated in 1992; however, the rate increased significantly to 17% in 1999 (p=0.03). (Tacconelli et al, 2002). In the United States, a tertiary care teaching hospital reported similar increases in rates of MDRPA despite different definitions used. (D'Agata, 2004). Among 2344 *P. aeruginosa* isolates, an increase in MDRPA (resistance to ceftazidime, ciprofloxacin, and aminoglycosides) from 0.9% in 1994 to 5.6% in 2002 was identified. The same study evaluated multidrug resistance (to imipenem, ceftazidime, and ciprofloxacin) in 1989 *P. aeruginosa* isolates and reported an increase from 0.6% in 1994 to 4.7% in 2002. Recently, another academic medical center in the United States reported an increase in MDRPA from 0% (0/18) in 1998 to 32% (13/41) in 2002 (p=0.0025).(Jung R, Fish DN, Obritsch MD, et al, 2004). Therefore, rates of resistance are increasing on a global scale, as well as within specific institutions. Institution-specific surveillance of susceptibility of *P. aeruginosa* isolates to determination of rates of multidrug resistance and antipseudomonal agents are essential in guiding therapy in individual patients.

3.1.6. Antibiotic Resistance

Pseudomonas aeruginosa is a very relevant opportunistic pathogen. One of the most worrisome characteristics of *P.aeruginosa* is its low antibiotic susceptibility. This low susceptibility is count to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes (e.g., *mexAB, mexXY* etc.) and the low permeability of the bacterial cellular envelopes. In addition to this intrinsic resistance, *P. aeruginosa* frequently develops acquired resistance either by mutation in chromosomally-encoded genes or by the horizontal gene transfers of antibiotic resistance determinants. Development of multidrug resistance by *P.aeruginosa* isolates requires several different genetic events such as acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes. Hypermutation helps the selection of mutation-driven antibiotic resistance in *P. aeruginosa* strains producing chronic infections, but the clustering of several different antibiotic resistance genes in integrons favors the concerted acquisition of antibiotic resistance determinants. Some recent

studies have concluded that phenotypic resistance associated to biofilm formation or to the emergence of small-colony variants may be important in the response of *P. aeruginosa* populations to antibiotics treatment (Cornelis, 2008).

3.2. Proteus

Proteus mirabilis is a Gram-negative, facultativelyanaerobic, rod shaped bacterium. It shows swarming motility, and urease activity. *P. mirabilis* causes 90% of all 'Proteus' infections in humans. *Proteus* species are of the Enterobacteriaceae family of gram-negative bacilli. *Proteus* organisms are implicated as serious reason of infections in humans, along with *Escherichia, Klebsiella, Enterobacter,* and *Serratia*species. *Proteus* species are mostly found in the human intestinal tract as part of normal human intestinal flora, along with *Escherichia coli* and *Klebsiella* species, of which *E coli* is the predominant resident. *Proteus* is also found in multiple environmental habitats hospitals. In hospital settings, it is not unusual for gram-negative bacillus to colonize both the skin and oral mucosa of both patients and hospital personnel. Infection primarily occurs from these reservoirs. However, *Proteus* species are not the most common cause of nosocomial infections. *Proteus mirabilis* causes 90% of *Proteus* infections also can be considered a community-acquired infection. *Proteus vulgaris* and *Proteus penneri* are isolated from individuals easily in long-term care facilities and hospitals and from patients with underlying diseases or compromised immune systems. Patients with recurrent infections, with structural abnormalities of the urinary tract, those who have had urethral instrumentation, and those whose infections were acquired in the hospital have an increased frequency of infection caused by *Proteus.*

3.2.1. Pathophysiology:

Proteus species having an extracytoplasmic outer membrane, a feature shared with other gram-negative bacteria. In addition, the outer membrane contains a lipid bilayer, lipoproteins, polysaccharides, and lipopolysaccharides. Infection depends on the interaction between the infecting organism and the host defense mechanisms. Various components of the membrane interplay with the host to determine virulence. Inoculum size is important and has a positive correlation with the risk of infection. Certain virulence factors have been identified in bacteria.

The first step in the infectious process is adherence of the microbe to host tissue. Fimbriae facilitate adherence and thus enhance the capacity of the organism to produce disease. *E coli, P mirabilis*, and other gram-negative bacteria contain pili, which are tiny projections on the surface of the bacterium. Specific chemicals located on the tips of pili enable organisms to attach to selected host tissue sites (eg, urinary tract endothelium). The presence of these fimbriae has been demonstrated to be important for the attachment of *P mirabilis* to host tissue. The adhesion of *Proteus* species to uroepithelial cells initiates several events in the mucosal endothelial cells, including secretion of interleukin 6 and interleukin 8. *Proteus* organisms also induce epithelial cell desquamation. Bacterial production of urease has also been shown to increase the risk of pyelonephritis in experimental animals. Urease production, together with the presence of bacterial motility and fimbriae, may favor the production of upper urinary tract infections (UTIs) by organisms such as *Proteus. Proteus* and *Pseudomonas* species are the microorganisms most commonly responsible for gram-negative bacteremia. When these pathogens enters the bloodstream, endotoxin, a component of gram-negative bacteria cell walls, apparently triggers a cascade of host inflammatory responses and leads to major detrimental effects.
As *Proteus* and *Pseudomonas* both are gram-negative, they can cause gram-negative endotoxin-induced sepsis, resulting in systemic inflammatory response syndrome (SIRS), which carries a mortality rate of 20%-50%. Although other organisms can trigger a similar response, it is useful to consider gram-negative bacteremia as a distinct entity because of its characteristic epidemiology, pathogenesis, pathophysiology, and treatment. The presence of the sepsis syndrome associated with a UTI should raise the possibility of urinary tract obstruction. This is especially true of patients who reside in long-term care facilities, who have long-term indwelling urethral catheters, or who have a known history of urethral anatomic abnormalities. The ability of *Proteus* organisms to produce urease and to alkalinize the urine by hydrolyzing urea to ammonia makes it effective in producing an environment in which it can survive. This leads to precipitation of organic and inorganic compounds, which leads to struvite stone formation. Struvite stones are composed of a combination of magnesium ammonium phosphate (struvite) and calcium carbonate-apatite. Struvite stone formation can be sustained only when ammonia production is increased and the urine pH is elevated to decrease the solubility of phosphate. Both of these requirements can occur only when urine is infected with a urease-producing organism

such as *Proteus.* Urease metabolizes urea into ammonia and carbon dioxide: Urea → $2NH_3$ + CO_2. The ammonia/ammonium buffer pair has a pK of 9.0, resulting in the combination of highly alkaline urine rich in ammonia. Symptoms attributable to struvite stones are uncommon. More often, women present with UTI, flank pain, or hematuria and are found to have a persistently alkaline urine pH (>7.0). The genitourinary tract is the place of disease responsible for gram-negative bacteremia in approximately 35% of patients. In previously healthy outpatients, *E coli* is by far the most often implicated cause of UTIs. In contrast, individuals with multiple prior infections of UTI, multiple antibiotic treatments, urinary tract obstruction, or infection developing after instrumentation frequently become infected with *Proteus* bacteria or other bacteria such as Enterobacter, *Klebsiella, Serratia,* and *Acinetobacter*. Bacteriuria occurs in 10%-15% of hospitalized patients with indwelling catheters. The risk of infection is 3%-5% per day of catheterization.

3.2.2. Multidrug resistance proteus mirabilis:

Isolates from Poland beach several bla_{CMY}s ($bla_{CMY\text{-}4,\ -12,\ -14,\ -15,\ -38}$ and a new $bla_{CMY\text{-}45}$), while isolates from Italy and Greece harbored $bla_{CMY\text{-}16}$ only. Earlier collected with $bla_{CMY\text{-}4}$ or $_{-12}$, recovered in France from Greek and Algerian patients, were also studied. All isolates showed striking similarities. . Moreover, modules were inserted into the same chromosomal site, within the *pepQ*gene. Since ColE1 plasmids carrying IS*Ecp1* with similar *C. freundii* DNA fragments (Tn*6114*) had been studied earlier, it is likely that a similar molecule had mediated at some stage this DNA transfer between *C. freundii* and *P. mirabilis*. Isolates with $bla_{CMY\text{-}12}$, $_{-15}$ and $_{-38}$ genes second bla_{CMY} copy within a shorter IS*Ecp1* module (Tn*6113*), always inserted downstream of the *ppiD* gene. Sequence analysis of all mobile $bla_{CMY\text{-}2}$-like genes shows that those integrated in the *P. mirabilis* chromosome form a distinct cluster that may have formed by the stepwise accumulation of mutations. All these observations, coupled to strain typing data, suggest that the bla_{CMY} genes studied here may have originated from a single IS*Ecp1*-mediated mobilization-transfer-integration process, followed by spread and evolution of a *P. mirabilis* clone over time and a large geographic area. **(**D'Andrea et al, 2011).

3.3. Antibiotics and their mechanism of action:

3.3.1. Tetracycline

Tetracyclines were discovered in the 1940s (Fig 1) and exhibited activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites. These are inexpensive antibiotics, which have been used extensively in the prophlylaxis and therapy of human and animal infections and also at subtherapeutic levels in animal feed as growth promoters. The initial tetracycline-resistant bacterium, Shigella dysenteriae, was isolated in 1953. Tetracycline resistance now found in an increasing number of pathogenic, opportunistic, and commensal bacteria. The presence of tetracycline-resistant pathogens limits the use of these antibiotics in treatment of disease. Tetracycline resistance is often due to the acquisition of transgenes, which code for energy-dependent efflux of tetracyclines or for a protein that protects bacterial ribosomes from the action of tetracyclines. Many of these genes are linked with mobile plasmids or transposons and can be distinguished from each other using molecular methods including DNA-DNA hybridization with oligonucleotide probes and DNA sequencing. A little number of pathogens acquire resistance by mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, change the regulation of innate efflux systems, or alter the 16S rRNA (Fig 2). New tetracycline derivatives are beingstudied, although their role in treatment is not clear. Changing the use of tetracycline in human and animal health as well as in food production is needed if we are to continue to use this class of broad-spectrum antimicrobials through the present century (Ian et al, 2011).

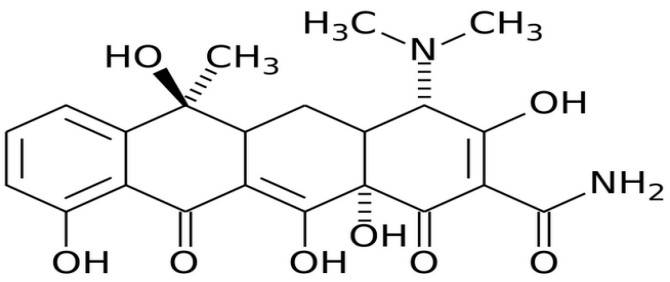

Fig 1: Tetracyclin chemical struture

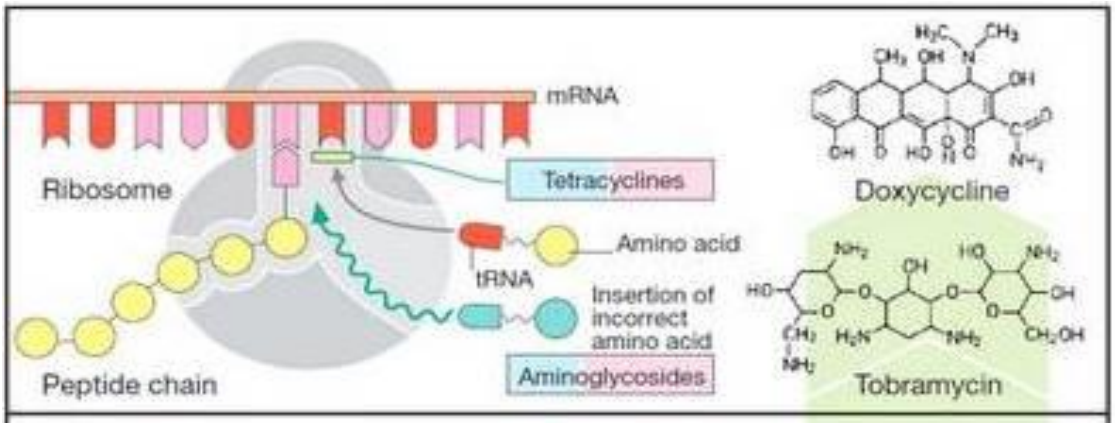

Fig 2: Tetracycline mode of action

3.3.2. Vancomycin: Vancomycin was first isolated (Fig 3) in 1953 by Edmund Kornfeld (working at Eli Lilly) from a soil sample collected from the interior jungles of Borneo by a missionary. (Shnayerson et al, 2003). The organism that produced it was initially named *Amycolatopsis orientalis*. (Levine et al 2006). The real use for vancomycin was for the treatment of penicillin-resistant strains. (Moellering et al, 2006) (Levine et al, (2006).

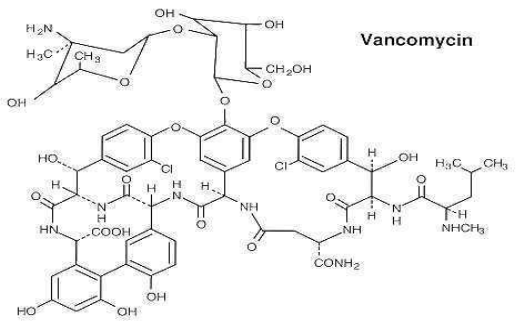

Fig 3: Vancomycin chemical structure

Mechanism of Action: Vancomycin is an antibiotic produced by *streptomyces orientalis***.** It is a complex chemical entity consisting of amino acids and sugars. Vancomycin inhibits peptidoglycan synthesis by binding the D-alanyl-D-alanine group on the peptide side chain of one of the membrane-bound intermediates (Fig 4). Vancomycin affects by inhibiting cell wall synthesis in Gram-positive bacteria. Due to the different mechanism by which Gram-negative bacteria form their cell walls and the various factors related to entering the outer membrane of Gram-negative organisms, vancomycin is not active against Gram-negative bacteria (except some non-gonococcal species of *Neisseria*). To specific, vancomycin prevents joining of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) peptide subunits into the cell wall peptidoglycan matrix; which forms the major structural component of Gram-positive cell walls. The large hydrophilic molecule are able to formation of hydrogen bond interactions within the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides. Under normal circumstances, it is a five-point interaction. This attachment of vancomycin to the D-Ala-D-Ala prevents the incorporation of the NAM/NAG-peptide subunits into the peptidoglycan matrix.

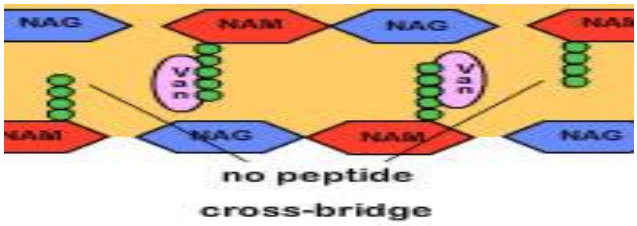

Fig 4: mechanism of action of vancomycin

3.3.3. Ciprofloxacin

Ciprofloxacin is a synthetic chemotherapeutic antibiotic (Fig 5) of the fluroquinolone drug class (Nelson et al, 2007), (Kawahara et al, 1998). It is a secondgeneration fluoroquinolone antibacterial. It stops bacterial infections by interfering with the enzymes that cause DNA to rewind after being copied,which stops synthesis of DNA and protein.

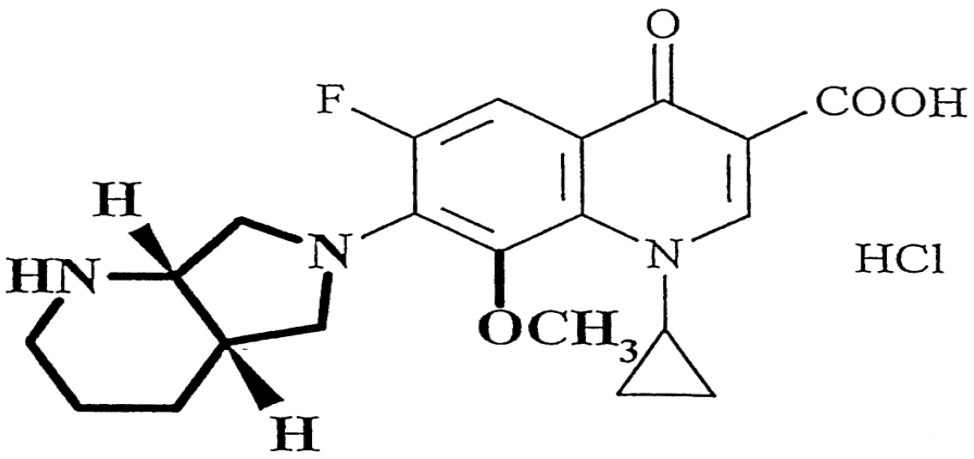

Fig 5: Ciprofloxacin chemical structure

Mechanism of action: Ciprofloxacin is one of the broad-spectrum antibiotic active against both Gram-positive and Gram-negativebacteria. It works by inhibiting DNA gyrase, topoisomerase IV and a type II topoisomerase (Drlica et al, 1997) enzymes necessary to separate bacterial DNA, thereby inhibiting cell division. This mechanism can also affect mammalian cell replication. In particular, some compounds of this drug family (for example those that contain the C-8 fluorine) (Robinson et al, 1992) display high activity not only against bacterial topoisomerases but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and *in vivo* tumor models. (Sissi and Palumbo, 2003). Although quinolones are very much toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known. Quinolone-induced DNA damage was first reported in 1986. (Hussy et al, 1986). Recent studies have shown a correlation between mammalian cell cytotoxicity of the quinolones and the induction of micronuclei. (Forsgren et al, 1987), (Gootz et al, 1990) As such, some fluoroquinolones also

cause injury to the genes of eukaryotic cells**.** (Elsea *et.al,* 1992), (Suto *et a,l* 1992), (Enzmann *et al,* 1999), There continues to be debate as to whether or not this DNA damage is to be considered one of the mechanisms of action concerning the severe adverse reactions experienced by some patients following fluoroquinolone therapy**.** (Sissi and Palumbo 2003), (Yaseen et al, 2003).

3.3.4. Amoxicillin

Amoxycillin is a moderate-spectrum, bacteriolytic, β-lactam antibiotic (Fig 6) used to treat bacterial infections caused by susceptible microorganisms. Usually the drug of choice in the class because it is comparatively better absorbed, following oral administration, than other β-lactam antibiotics. Amoxicillin is the most common antibiotics prescribed for children. Amoxicillin is sensitive to degradation by β-lactamase-producing bacteria, which are resistant to a broad spectrum of β-lactam antibiotics, such as penicillin. For this cause, it is often mixed with clavulanic acid, a β-lactamase inhibitor, and marketed under one name. This upgiven effectiveness by reducing its susceptibility to β-lactamase resistance.

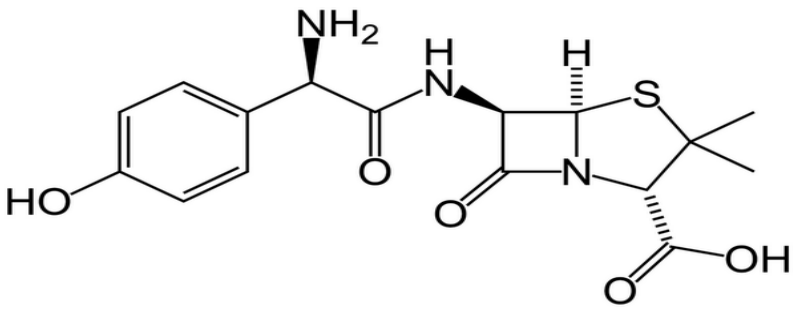

Fig 6**:** Amoxicillin chemical structure

Mechanism of action: This drug works by preventing the formation of bacterial cell walls. It prevents cross-linkage among the linear peptidoglycan polymer chains that make up a major component of the cell walls of both Gram-positive and Gram-negative bacteria. It has

two ionizable groups present in between the physiological range (the amino group in alpha-position to the amide carbonyl group and the carboxyl group).

3.3.5. Chloramphenicol

Chloramphenicol is a bacteriostatic antimicrobial (Fig 7). It is a most common broad-spectrum antibiotic, alongside the tetracycline. Chloramphenicol is effective against a variety of Gram-negative and Gram-positivebacteria, including most anaerobic organisms. It prevents the peptidyl bond formation between the amino acids of growing polypeptide chain. (Fig 8).

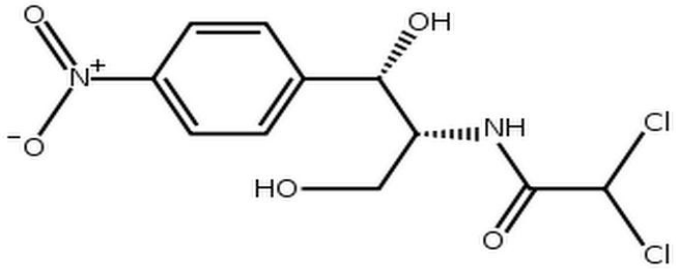

Fig 7: chloramphenicol chemical structure

Mechanism of action:

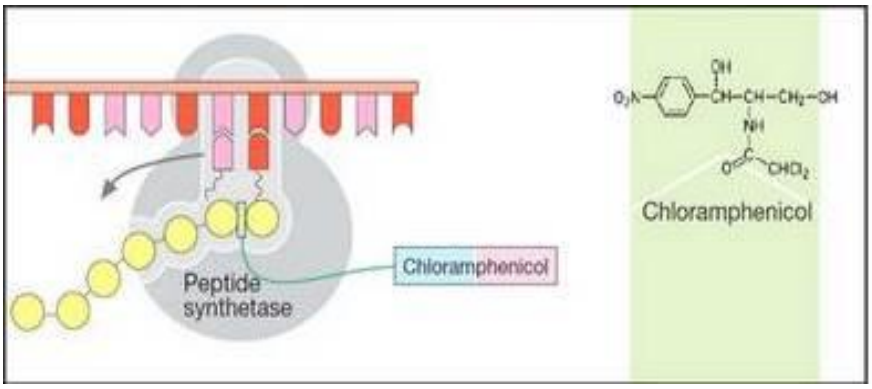

Fig 8: mode of action of chloramphenicol

4.1. COLLECTION OF SAMPLE

Pathogenic sample was collected from the hospital, Ispat General Hospital, Rourkela.

4.2. ISOLATION OF PURE CULTURE

Pure cultures of the pathogens were isolated by using nutrient agar plate. From the pathogenic bacteria vial by streaking on the nutrient agar medium pure isolated colonies of pathogenic bacteria can be obtained.

Further expements such as antibiotic susceptibility test and minimum inhibitory concentration analysis was done by using Muller-Hinton agar and Muller-Hinton broth respectively.

4.3. BIOCHEMICAL IDENTIFICATION TESTS

Indole produdtion test: The indole test is a biochemical test tested on bacterial pathogenic species to determine the ability of the organism to split indole from the amino acid tryptophan. This division is produced by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase".

Principle

Indole is produced by a reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase enhances the deamination reaction, during which the amine (NH2) group of the tryptophan molecule is removed. Final products of the reaction are pyruvic acid, indole, ammonia (NH3) and energy. The indole produced during the reaction is detected by the addition of Kovac's reagent (dimethylaminobenzaldehyde) which produces a cherry-red reagent layer. A positive result was the presence of a red or red color in the surface alcohol layer of the broth. A negative result appears yellow. Variable result may also occur, showing an orange color as a result.

Methyl red test: The Methyl-Red test tests for the importance to perform mixed-acid fermentation.MR-VP broth contain glucose, peptone, and a phosphate buffer. Organisms that

perform mixed-acid fermentation produce sufficient acid to jumpover the buffering capacity of the broth, so a decrease in pH results. Organisms that perform other kinds of fermentation cannot crossover the buffering capacity of the broth. After incubation, the pH indicator Methyl Red was added to the broth. Methyl Red shows red at pH below 4.4 (this may be a positive result) and yellow at pH above 6.0. An orange color indicates an intermediate pH and may be a negative result.

4.4 McFarland standard:

McFarland standards are extensively used as turbidity standards for the preparation of suspensions of microorganisms. The McFarland 0.5 standard has various application in the preparation of bacterial inocula for performing antimicrobial susceptibility testing. One of the earliest uses of turbidity for the enumeration of bacterial populations was in the preparation of vaccines (Lorian, 1986). In 1907 McFarland developed a number of barium sulfate solutions to approximate the numbers of bacteria in solutions of equal turbidity, as determined by plate counts (McFarland, 1907) (Forbes et al, 1998). The capability of susceptibility testing requires the use of standard inocula. The McFarland 0.5 standard was used for the preparation of inocula in standardized agar dilution, broth macro- and microdilution, disc diffusion and anaerobic organism susceptibility test procedures.

4.4. PRINCIPLES OF THE PROCEDURE

Turbidity standards are prepared by adding homogenously chemicals that precipitate to form a solution of reproducible turbidity.3 McFarland standards are prepared by adding sulfuric acid to an aqueous solution of barium chloride, which results in the formation of a suspended barium sulfate precipitate. The McFarland 0.5 standard corresponds approximately to an homogeneous *Escherichia coli* suspension of 1.5 x 108 cells per mL. (Forbes et al, 1998).

REAGENTS:

McFarland Turbidity Standard No. 0.5

Approximate Formula Per 100 mL Purified Water

Sulfuric Acid, 0.18 M . . . . . 99.5 mL

Barium Chloride, 0.048 M . . . . . 0.5 mL

Vigorous agitation using a mechanical vortex mixer, density was checked for the turbidity standard by determining the absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.10.

USE:

Use of the McFarland 0.5 standard will enable the preparation of standardized inocula for use in the performance of standardized antimicrobial susceptibility testing procedures.

4.5. Antimicrobial susceptibility test:

Disc diffusion method

Modified Kirby-Bauer disk diffusion method was used to test the susceptibility of the *Proteus* and pseudomonas isolates to different antimicrobial agents: ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), vancomycin (30 μg), kanamycin (30 μg), and ciprofloxacin (30 μg), The inocula were prepared by growing the various *Proteus* species on separate agar plates and colonies from the plate were transferred with inoculating loop into 3 ml of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Muller-Hinton agar plate was evenly inoculated with the organisms using a sterile swab. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid. The wet swab was then used to inoculate the Muller-Hinton agar, evenly streaked across the surface. By means of Disc Dispenser the antibiotic discs were applied to the surface of the inoculated agar and the plates were incubated overnight at 37 ^{0}C. The diameter of zone of

inhibition was observed and measured and compared to the chart provided by National Committee for Clinical Laboratory Standards (NCCLS).

4.6. PROCEDURE:

Muller-Hinton Agar was prepared.

From the standardized inoculums prepared by using 0.5 McFarland solutions was then Spreaded over the media surface using a clean sterilized cotton swab.

Different antibiotic disks were placed in each plate with proper spacing using a sterile forcep.

Now the plates were kept for incubation at 37^0C for 24 hour for proper growth.

Antibiotics to which organisms are sensitive, formed clear zone around it, and to which organisms are resistance, they do not form any zone of inhibition around it. Those that are intermediate, forms a little zone of inhibition.

Diameter of the zone was measured ,compared with the standard zone diameter given in the protocol chart it can be determine that whether the strain is resistance or intermediate or susceptible toward the antibiotic.

4.7. PREPARATION OF SERIALLY DILUTED ANTIMICROBIAL AGENT:

The antimicrobial agents are diluted to a number of times with ratio 1:1. The concentration of antibiotic in the solution is in microgram/mL.

The quantity of antimicrobial agent to be taken for the preparation of a known concentration of antibiotic solution was carried out by using the following formula.

$$\text{WEIGHT (mg)} = \frac{\text{VOLUME (mL)} * \text{Conc. (µg/mL)}}{\text{POTENCY (µg/mg)}}$$

Biochemical identification test:

Pseudomonas: Biochemical analysis shows that this strain of *pseudomonas* is gram negative, aerobic rod, the species identified in the biochemical identification (Fig 9) analysis is *pseudomonas aeruginosa*..

Fig -9 Biochemical test result for *Pseudomonas*

Proteus: Biochemical analysis shows (Fig 10) that the strain of *proteus* identified is *proteus mirabilis.*

Fig-10 Biochemical test result for *Proteus*

Table 1: Enterobacteriaceae Identification test

Sl.No.	Test	*proteus*	*Pseudomonas*
1	ONPG	-	-
2	Lysine utilization	-	+
3	Ornithine utilization	-	+
4	Urease	-	-
5	Phenylalanine deamination	-	+
6	Nitrate reduction	+	+
7	H_2S production	-	+
8	Citrate utilization	+	+
9	Voges proskauer's	+	-
10	Methyl red	+	-
11	Indole	-	+
12	Malonate utilization	-	+

Sl.No.	Test	*proteus*	*pseudomonas*
13	Esculin hydrolysis	+	+
14	Arabinose	-	+
15	Xylose	-	+
16	Adonitol	-	-
17	Rhamnose	-	-
18	Cellobiose	+	-
19	Melibiose	-	-
20	Saccharose	+	-
21	Raffinose	-	-
22	Trehalose	+	+
23	Glucose	+	+
24	Lactose	+	-
25	Oxidase	+	-

Table 2: Carbohydrate Utilization Test

Sl NO.	TEST	*Proteus*	*pseudomonas*
PART A			
1	Lactose	-	-
2	Xylose	-	+
3	Maltose	+	-
4	Fructose	+	-
5	Dextrose	+	-
6	Galactose	+	+
7	Raffinose	-	-
8	Trehalose	+	-
9	Melibiose	-	-
10	Sucrose	+	-
11	L- arabinose	-	+
12	Mannose	+	+
PART B			
13	Inulin	+	-
14	Sodium gluconate	+	-
15	Glycerol	-	-
16	Salicin	+	-

17	Dulcitol	-	-
18	Inositol	-	-
19	Sorbitol	+	-
20	Mannitol	+	-
21	Adonitol	-	-
22	Arabitol	-	-
23	Erythritol	-	-
24	α – methyl –D-glucoside	-	-
PART C			
25	Rhamnose	-	-
26	Cellobiose	+	-
27	Melezitose	+	-
28	α –methyl-D-mannoside	-	-
29	Xylitol	-	+
30	ONPG	-	-
31	Esculin hydrolysis	+	+
32	D-Arabinose	-	+
33	Citrate utilization	-	+
34	Malonate utilization	-	+
35	Sorbose	-	-

Table -3: Antibiotic disk diffusion test:

	Tetracycline (30 mcg)	Vancomycin (30 mcg)	Ciprofloxacin (30 mcg)	Amoxicillin (30 mcg)	Chloramphenicol (30 mcg)	Kanamycin (30 mcg)
pseudomonas	Resistance	Resistance	Resistance	Resistance	Resistance	Resistance
Proteus	Resistance	Sensitive	Resistance	Sensitive	Resistance	Resistance

As *pseudomonas* is resistance to the entire antibiotic hence no zone of inhibition was formed (Fig 11).

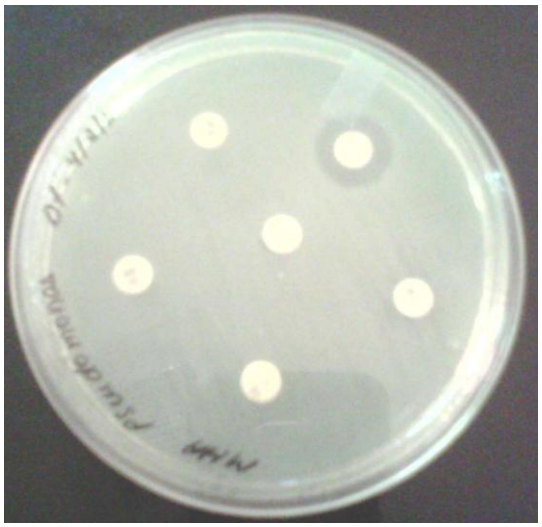

Fig-11 *Pseudomonas* antibiotic disc diffusion test

Very small zone of inhibition was formed around amoxicillin and vancomycin, but no zone formation around other antibiotics (Fig 12).

Fig-12 *proteus* antibiotic disc diffusion test

1. *Pseudomonas* shows resistance towards most antibiotics.
2. *Proteus* shows sensitive towards some antibiotic like vancomycin and amoxicillin.
3. Vancomycin and amoxilin are least effective toward these microorganisms.
4. Tetracycline, ciprofloxacin, chloramphenicol and kanamycin are most effective toward these microorganisms.

Table 4: Antibiogram of disk diffusion technique

organism	antibiogram
Pseudomonas	$T^R V^R Cf^R Am^R C^R K^R$
proteus	$T^R V^S Cf^R Am^S C^R K^R$

MIC RESULT: Concentration of antibiotic solution=µg/mL

MIC is the minimum concentration of antibiotic at which the bacteria shows a remarkable inhibition.

Table -5: Minimum inhibitory concentration

	Vancomycin	Penicillin	Chloramphenicol	Amoxicillin
pseudomonas	32	8	16	Resistance
proteus	16	4	Sensitive	Sensitive

1. *Pseudomonas* is resistance toward amoxicillin.
2. *Proteus* is sensitive toward chloramphenicol and amoxicillin.

DISCUSSION:

Mode of resistance to antimicrobials used for the treat infectious disease has been known since before antibiotics were introduced into routine clinical usage (Abraham and Chain 1940). Imprudent and often regular administration of antimicrobials has, however, compounded the problem by enriching for resistant bacteria populations at the expense of sensitive ones (Lerner 1998; Hellinger 2000; Livermore 2000). With all too increasing frequency human pathogens *proteus* and *pseudomonas* displaying resistance to multiple antimicrobials, and the attendant challenge of treating infections with an ever-dwindling number of effective therapeutic agents. Pathogen shows resistance to antimicrobial agents as they are mutating their genome or acquiring some resistance mechanism. Administration of antibiotics for prolonged period helps in increasing the resistance mechanism of the pathogens. Multi drug resistance *Pseudomonas aeruginosa* increases its resistance capacity and potency to a higher extent. So some potential drug should be made for the treatment of *P.aeruginosa* infections. Similarly: The Proteus species isolated were found to have high antimicrobial resistance against tetracycline, chloramphenicol. *Proteus* also acquiring resistance to multiple drugs. It is susceptible to some antibiotics like chloramphenicol, vancomycin, and amoxicillin. Regular drug administration to these strains would acquire multi-drug resistance property. *Pseudomonas aeruginosa* has became increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several epidemiological studies shows its occurrence as a nosocomial pathogen and indicate that antibiotic resistance is increasing in clinical isolates *Pseudomonas* shows resistance toward most antibiotics. The minimum inhibitory concentration of antibiotics toward *pseudomonas* is very high.

FUTURE PROSPECTS

In light of current problems with multiple antibiotic resistances in clinical strains and the potential for increasing resistance to antibiotic whose use is increasing in the community, it is clear that prudent use of available and as yet effective antimicrobials is called for. Nonetheless, resistance is a common theme in infection and one that is unlikely to disappear soon. For this reason, the targeting of resistance mechanisms themselves was gaining ability(Wright 2000; Poole 2001c), with more generalized resistance mechanisms such as membrane impermeability and multidrug efflux possibly favoured targets. The first inhibitors of the broadly specific

multidrug efflux systems of *Ps. aeruginosa* have recently been reported in the literature (Renau et al. 1999; Lomovskaya et al. 2001). Effective at overcoming existing resistance to, e.g. fluoroquinolones, these agents are also effective at preventing the emergence of fluoroquinolone resistance in the first place (Lomovskaya et al. 2001). They are likely,to be highly effective at compromising intrinsic and acquired biocide (i.e. triclosan) resistance in this organism as well as antibiotic and biocide resistance in organisms expressing homologous multidrug efflux systems (Blais et al. 1999). Inhibitors of the NorA multidrug transporter of *Staph. aureus* have also been reported (Schmitz et al. 1998; Aeschlimann et al. 1999; Markham et al. 1999; Guz et al.2001) where they are effective at enhancing fluoroquinolone susceptibility (Schmitz et al. 1998; Aeschlimann et al. 1999; Markham et al. 1999) and preventing emergence of fluoroquinolone resistance in vitro (Markham and Neyfakh 1996). Similarly, enhancement of antimicrobial susceptibility using agents that permeabilize, e.g. the outer membrane has shown some challenging promise, for specific antimicrobials (Ayres et al. 1998; Savage 2001; Poole 2001). Finally, owing to the prevalence of the biofilm mode of bacterial growth in vivo and its importance vis-a`-vis antibiotic and biocide insusceptibility, strategies aimed at interfering with biofilm formation/ function are also likely to be useful in countering clinical episodes of antibiotic and biocide resistance. Natural susceptibility in populations of disease-causing bacteria through the prudent use of current and future antimicrobials. Compounds that interfere with bacterial cell signaling processes necessary for biofilm formation have now been identified (Manefield et al. 1999; Rice et al. 1999; Borchardt et al. 2001) and could be useful in blocking biofilm formation or maintenance. Chemical (Armstrong et al. 2000) and electromagnetic (Blenkinsopp et al. 1992; McLeod et al. 1999) approaches to biofilm disruption have also been reported. Still, the best hope of controlling infectious diseases is and will continue to be maintaining susceptibility in populations of disease-causing bacteria through the prudent use of current and future antimicrobials.

APPENDIX I

Nutrient agar

Ingredients Gms / Litre

Peptic digest of animal tissue 5.000

Sodium chloride 5.000

Beef extract 1.500

Yeast extract 1.500

Agar 15.000

Final pH (at 25°C) 7.4±0.2

Muller-Hinton agar

For 1 liter of medium:

- Acid hydrolysate of casein .....17.5 g

- Beef infusion .....2.0 g

- Soluble starch .....1.5 g

- Bacteriological agar .....17.0 g

pH of the ready-to-use medium at 25°C : 7.3 ± 0.2

APPENDIX II

0.5 McFarland standard

Sulphuric Acid ,1% 995.00 ml

Barium Chloride, 1% 5.00

Kovac's reagent

p-Dimethylaminobenzaldehyde 7 4.8%

Isoamyl Alcohol 4.8%

Hydrochloric Acid 23.8%

TDA reagent

Ferric chloride 10g/100ml (in distilled water)

Baritt reagent

Methyl reagent

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