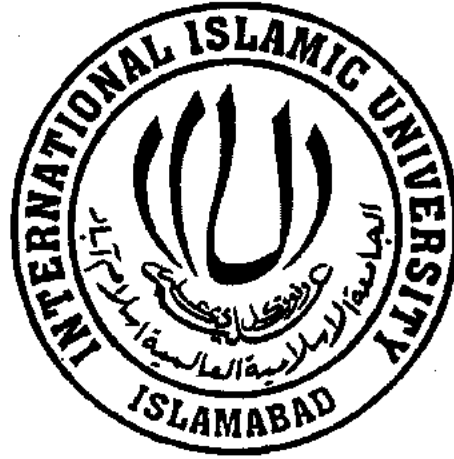# Molecular Identification and Characterization of *Pseudomonas* Strains Isolated From Clinical Specimens

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INTERNATIONAL ISLAMIC UNIVERSITY, ISLAMABAD, PAKISTAN

Molecular Identification and Characterization of *Pseudomonas* Strains Isolated From Clinical Specimens

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A dissertation submitted as partial fulfillment of requirements for the degree of MS in

Biotechnologyat the Faculty of Basic and Applied Sciences

International Islamic University Islamabad, Pakistan

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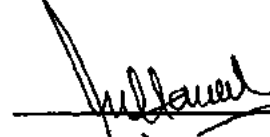*International Islamic University Islamabad, Pakistan*

بسم الله الرحمن الرحيم

FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Nagina Atlas and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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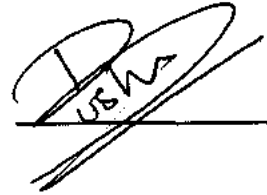**Internal Examiner**

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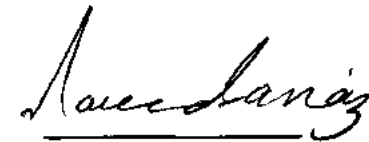
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as a partial fulfillment for the award of the degree of

MS Biotechnology

Dedication

This work is dedicated to my Adday and Baba Jee

Declaration

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date _____

Nagina Atlas

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Contents

Acknowledgement

In the name of Allah, The Most Beneficent, The Most Merciful

Thanks to **ALLAH** Almighty who is the entire source of knowledge and wisdom endowed to mankind, enabled me to carry out my research work and gave me the courage and patience to carry out this work. All praises to our **Last Prophet Hazrat MUHAMMAD** (Peace Be upon Him), who is forever a torch of guidance and knowledge for humanity as a whole

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Nagina Atlas

I

LIST OF ABBREVIATIONS

P   *Pseudomonas*

PCR   Polymerase Chain Reaction

µg   Microgram

µl   micro litter

mM   Milli Molar

mg   Milli gram

ml   Milli liter

DNA   Deoxyribonucleic acid

EDTA   Ethylene diamine tetra acetic acid

SDS   Sodium Dodecyl Sulphate

MDRPA   Multidrug resistant *Pseudomonas aeruginosa*

MRSA   Mithicillin Resistant *Staphylococcus aureus*

%   Percentage

°C   Centigrade

RPM   Revolution per minute

TBE   Tris base boric acid ethylene diamine tetrs acetic acid

TE   Tris ethylene diamine tetra acetic acid

Psi   Pounds per square inch

bp   Base pair

pH   Hydrogen ion concentration

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ABSTRACT

Molecular analysis of bacterial 16S rRNA genes has made a significant contribution to the identification and characterization of bacterial flora. In this study we have used 16S universal primer to investigate and characterize important *Pseudomonas* strains isolated from clinical samples. In our study, 40 isolates of *Pseudomonas* were recovered from total 50 clinical samples. Origin of the samples was pus, blood, urine and wounds swabs. Identification of *Pseudomonas* strains was done on the basis of morphological and biochemical testing. All those strains which were declared as *Pseudomonas* were screened for the antimicrobial compound production against three samples of methicillin-resistant *Staphylococcus aureus* (MRSA) and two environmental isolated bacteria by using Agar well diffusion method and Chloroform Overlay Assay. Antibiotics susceptibility of antimicrobial compound producing *Pseudomonas* strains was also determined. Those samples which produced highest zones of inhibition against all the tested microorganisms were further sequenced and the query sequences were blasted against the available sequences by using BLAST program on NCBI. In the end, all the sequenced samples were declared as *Pseudomonas aeruginosa*. Phylogenetic tree was constructed on the basis of 16S rRNA gene sequence of six samples and reference strain by using neighbor joinging method. Reliability of phylogenetic tree was estimated by bootstrapping phylogenetic reconstruction of 1000 replicates. The results of our study showed that *Pseudomonas aeruginosa* is producing antimicrobial compound against methicillin-resistant *staphylococcus aureus* (MRSA) and environmental isolated bacteria. This compound can be used as a good alternative to treat and control MRSA infections.

INTRODUCTION

1. INTRODUCTION

Pseudomonas aeruginosa is the most prevalent opportunistic human pathogen, and the most widespread Gram-negative bacterium found in nosocomial infections. Regardless of advances in antimicrobial agents, *Pseudomonas aeruginosa* is increasingly becoming multi antibiotics resistant (Ana *et al.*, 2002). Its saprophytic nature is associated with warm moist situations in the human environment.

Pseudomonas aeruginosa transmission from healthy carriers or environmental sites can only occur in favorable conditions e.g. transmission to immune compromised patients occur by the hands of nurses or respirators. Risk of transmission increases with the length of stays in hospital e.g. human faecal carriage of *Pseudomonas aeruginosa* is usually around 3%; however, after 3 weeks carriage increases to 30%, and thus can also result to endogenous infection (Anzai *et al.*, 2000).

Healthy adults are seldom affected by *Pseudomonas aeruginosa* but in the last few decades their deadly infection has renowned this organism as the main cause in a variety of severe infections in hospitalized patients with suppressed immunity (Bonomo and Szabo, 2006).

In the last few decades, nosocomial bacterial pathogens resistant to almost all available antibiotics, namely 'Superbugs', become a challenge in therapeutic choices. Opportunistic pathogens, primarily those with the ability to acquire resistance and high level resistance to all antimicrobial agents is a matter of great concern for microbiologists regarding treatment, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Neu, 1983; Ami *et al.*, 2008).

Pseudomonas aeruginosa, A. baumannii and *Klebsiella pneumonia* are the three common Gram's negative bacteria which are resistant to multiple antibiotics. Among these *Pseudomonas aeruginosa* is the most commonly isolated pathogen and a vital aethiological agent in fatal respiratory tract infection, urinary tract, surgical site and infections in patients from intensive care units (Gaynes and Edwards, 2005). A high mortality rate is carried by Endocarditis and septicemia, more than 70% in immunocompromised patients by burns, cancer or patients addicted to drugs. In cystic fibrosis patients *Pseudomonas aeruginosa* has a role in causing chronic debilitating respiratory infections due to mucoid strains which brings casualty (Collee, 1996).

1.2 Habitat

The simple nutritional requirements of *Pseudomonas* species makes the genus to occur in many natural habitats like fresh water, soil, marine environments etc (Franzetti and Scarpellini, 2007). In hospitals they contaminate nebulizers, dialysate fluids, saline, on catheters and other diagnostic and therapeutic devices. There are some important nutritional substrates e.g. dissolved carbon dioxide and residual sulfur, phosphorus, iron and divalent cations as carbon which help them in their survival in distilled water. It can survive when treated with chlorohexidine and quaternary ammonium compounds (Borriello *et al.*, 2007).

1.3 Background

The genus *Pseudomonas* is the diverse and ecologically important group of bacteria that belongs to order *Pseudomonadales* and the family Pseudomonadaceae. Other genera of this family include *Azomonas, Azomonotrichon, Azorhizophilus, Azotobacter, Cellvibrio, Mesophilobacter, Rhizobacter, Rugamonas,* and *Serpens.*

Pseudomonas is wide spread, Gram-negative motile aerobic rods that characterized by an eminent metabolic adaptability (Skerman *et al.*, 1980; Cornelis, 2008; Franzetti and Scarpellini, 2007).

The word Pseudomonad is derived from the Greek words *pseudo* and *monas. Pseudo* means false and *monas* means a single unit. In the early history of microbiology the word "monad" was used to represent single-celled organisms. One of the claims about the species name *aeruginosa* is that the *aeruginosa* word may be derived from combination of two Greek words *ae* and *ruginosa. Ae-* means old or aged, and the *ruginosa* means rough (Brown, 1956).

Palleroni placed the *Pseudomonads* into five ribosomal RNA homology groups on the basis of rRNA and DNA correlation. On the basis of phenotypic characteristics, Gilardi separated it into seven major groups: namely, alcaligenes, fluorescent, pseudomallei, stutzeri, acidovorans, facilis- delafieldii, and diminuta.

Pseudomonas was first secluded from a blue-green discharge on surgical dressing by a French military surgeon Sedillot in 1850 (Winn *et al.*, 2006). The green blue color seen in many clinical isolates is because of the *aeroginosa* colonies (Huw *et al.*, 2010).

1.4 Morphology

Pseudomonas aeruginosa is straight or lightly elliptical rods, measuring 0.5 to 1.0μm in width and 1.5 to 5.0μm in length. They are aerobic, nonspore forming, and have one or more polar flagella. They are either unable to utilize carbohydrates as energy source or degenerate them "oxidatively" rather than fermentative pathway (Forbes *et al.*, 2007), devoid of gas formation (Ana *et al.*, 2002).

1.5 Cultural Characteristics

They produce large, pigmented colonies on nutrient agar while on 5% sheep blood agar they produce large flat spreading, mucoid, rough, pigmented colonies with characteristic metallic sheen. Many strains have 2-aminoacetophenone due to which they may produce a fruity, sweet, musty smell. They produce non lactose fermenting colonies with green pigmentation and metallic sheen on MacConkey agar. Colonies separated from respiratory tract infection samples produce huge amounts of alginate, an exopolysaccharide consisting of mannuronic and guluronic acids which helps in forming mucoid colonies (Forbes *et al.*, 2007; Winn *et al.*, 2006; Borriello *et al.*, 2007).

1.6 Biochemical Reaction

They are oxidase and catalase positive, can tolerate temperature range upto 42°C, OF glucose oxidatively utilized by the organism, reduces nitrate to nitrite, unable decarboxylate lysine and ornithine, but dihydrolyze agrinine, mannitol not fermented, motile, in TSI test they produce alkaline slant/alkaline but with no gas and no H_2S, indole negative, utilize citrate, unable to hydrolyze urea, do not produce phenyl pyruvic acid, liquefies gelatin, do not hydrolyze aesculin and utilize acetamide (Forbes *et al.*, 2007; Borriello *et al.*, 2007; Winn *et al.*, 2006; Collee, 1996).

The discrimination and differentiation of *Pseudomonas aeruginosa* can be done by using medium Cetrimide agar because Cetrimide inhibits most bacteria and acts as detergent. It increases pyocyanin and pyoverdinec production. However, 4% *Pseudomonas aeruginosa* strains isolated from clinical samples are unable to produce pyocyanin.

1.7 Virulence Factors

Several metabolites of *Pseudomonas aeruginosa* act as virulence factors and are helpful in colony development and infection of the host tissue. Virulence factors include endotoxin e.g. liopopolysaccharides (LPS) and several exotoxins such as lecithinase, proteases, haemolysins, elastase and DNase. Exotoxin A is considered as the most important exotoxin because its function is similar to diphtheria toxin which blocks protein synthesis. Certain structural components such as pili and capsule also act as virulence factors.

Despite all these virulence factors, *Pseudomonas aeruginosa* infects immunocompromised patients and it is still considered as an opportunistic bacteria. These virulence factors have an individual and significant role in infected site. For example: Proteases have an important role in corneal ulceration. Exotoxin and proteases (elastase) play its harmful role in burns infections and septicemia. In chronic pulmonary colonization a polysaccharide polymer Alginate and quorum sensing molecules have a role in biofilms development. Pyochelin and fluorescein act as vital bacterial siderophores. In vivo activity of fluorescein showed that it competes with mammalian iron-binding proteins such as transferring (David *et al.*, 2007).

1.8 Motility And Biofilm Formation:

Pseudomonas aeruginosa exhibits three modes of surface motility: swarming (Hocquet *et al.*, 2007; Shaid and Malik, 2005; Lister, 2002), twitching (Neu, 1983; Chatzinikolaou *et al.*, 2000; MacArthur *et al.*, 2004), and sliding ((Jayakumar *et al.*, 2007). Biofilm development and pathogenesis are linked with twitching and swarming motilities, the flagellum and type IV pili (TFP), are considered as virulence factors (Jayakumar *et al.*, 2007; Shaid and Malik, 2005). TFP are present at one pole of the bacteria and composed of pilus filaments. Twitching motility controls the cell extension and retracts the pili, enabling the cells to move (Chatzinikolaou *et al.*, 2000; MacArthur *et al.*, 2004). TFP are important for twitching motility. A few reports have suggested that they are important for swarming behavior (Hocquet *et al.*, 2007; Shaid and Malik, 2005), while other studies suggest that they are not essential (Bryan *et al.*, 1983; Kang *et al.*, 2003; Cao *et al.*, 2004). Sliding is considered as surface motility, independent of flagella and TFP although few details are present about it (Jayakumar *et al.*, 2007).

Most of the *Pseudomonas* species produces exopolysaccharides known as slime layers. Exopolysaccharides enable *Pseudomonas* to avoid phagocytic ability of mammalian white blood cells. Slime production is also important in biofilms developments which are not easy to eradicate from surfaces (Connie *et al.,* 2006).

Biofilms are three dimensional surfaces attached structures in which bacteria are implanted in a matrix containing polysaccharide, protein and DNA. In nature and infectious disease, it is known as favored form of bacterial growth, particularly, *Pseudomonas aeruginosa* infections in Cystic fibrosis. One of the important effects of biofilm is its resistance to antimicrobial agents (Davies *et al.,* 2009). Some studies regarding biofilms suggests that *Pseudomonas aeruginosa* are metabolically inactive within biofilms and its growth is much lesser than cells at the biofilm periphery, which may contribute to increase biofilm tolerance to antibiotics since metabolically active cell are often targeted by antimicrobials agents.

Few suggests that, *Pseudomonas aeruginosa* containing biofilms, isolated from cystic fibrosis patients are anaerobic, since many antimicrobial agents are less active under anaerobiosis therefore biofilm is important for the development antimicrobial resistance. Oxygen limitation has been shown to have a significant role in antimicrobial resistance of in vitro-grown *Pseudomonas aeruginosa* biofilms (Borriello *et al.,* 2004).

Biofilm formation is induced by aminoglycosides in *Pseudomonas aeruginosa*. Two genes required for this process are, gene *arr* and the second gene is *ndvB*. A study conducted by Lusara, *et al.,* 2004, to find out the link between biofilm development and antibiotic resistance phenotypes in *Pseudomonas aeruginosa* isolated from clinical samples. This study was conducted in Mexico and they assessed the biofilm forming ability of different phenotypes using 162 clinical *Pseudomonas aeruginosa* isolates. Only in 14% of strains biofilm was produced after 8 hours incubation and 8% after 24 hours. The 8% strains showed greater multidrug resistance, mostly to antibiotics Piperacillin-Tazobactam and Imipenem (Delissalde and Aibile, 2004)

1.9 Infections and Risk Factors of MDR *Pseudomonas Aeruginosa*

Some of these species, such as *Pseudomonas aeruginosa*, are opportunistic pathogens and it affects both plants and metazoans. Cystic fibrosis patients, burn victims, cancer patients, and

patients who are in intensive care units for extended periods of time are most at risk for *Pseudomonas aeruginosa* infections (Vrankrijker *et al.*, 2010; Williams *et al.*, 2010).

Infections caused by *Pseudomonas aeruginosa* ranked second most among Gram-negative infectious bacteria. This survey was reported by the United States national nosocomial infection surveillance system (Khan *et al.*, 2008).

Pseudomonas aeruginosa can cause infections almost at any external organ and it prefers moist sites e.g. tracheostomies, indwelling catheters, burns, and the external ear and weeping cutaneous wounds. It causes severe urinary tract and lower respiratory tract infections in immune-compromised hosts. This also causes *Pseudomonas* keratitis, infections of corneal ulcers, endophthalmitis can be fulminant and results into permanent blindness, endocarditis, meningitis, brain abscess, infections of bone and joints from hematogenous spread (Bielecki *et al.*, 2008).

Pseudomonas aeruginosa is the third frequent cause of bacteremia after *Esherichia coli* and *Klebsiella pneumoniae*. It causes 6% of all bacteremias and 75% of nosocomial bacteremias.

In a study by Shanthi *et al.*, 2009 the general sites affected by *Pseudomonas aeruginosa* were respiratory tract, urinary tract, wound and blood.

On experimental animals multidrug resistant *Pseudomonas aeruginosa* was also found to stimulate apoptosis of blood monocytes (Antonopoulo *et al.*, 2007). Multidrug resistant *Pseudomonas aeruginosa* is able to cross the intestinal epithelial layer and result into fatal sepsis by disrupting epithelial barrier function mechanism in immunocompromised patients.

Shankar *et al.*, 2005 conducted a study on bacterial etiology of diabetic foot infections in South India. They found that frequency of MDR *Pseudomonas aeruginosa was 44%* and gram negative bacteria are more prevalent (57.6%) than gram positives (42.3%).

Lodise *et al.*, 2007 found that in patients of respiratory tract infections, multidrug resistance is more complicated. The prevalence of MDRPA was 36%, Out of 351 patients of *Pseudomonas aeruginosa*. Most of the patients were male and the mean age was 60.5 ± 18.9yrs. Patients median hospital stay was 24 days and at the culture sample collection, 82% of patients were in ICU. The isolated strains were highly sensitive to amikacin, cefepime and tobramycin. In this study, they also discussed that chance of MDR *Pseudomonas aeruginosa* infections increased with high consumption of antibiotic.

According to the study Kohanteb *et al.*, 2007 frequency of multi drug resistant *Pseudomonas aeruginosa* was high in burn infections as compared to nosocomial pneumonia. They found out

that all the isolates from burn infection were resistant to antibiotics gentamycin, cephalothin carbencillin and ceftazidime. The lowest resistance level was showed by meropenem antibiotic.

A study was conducted by Jayakumar *et al.*, 2007 on prevalence of MDRPA with respect to ESBL and MBL in a tertiary care hospital. In this study they observed that the prevalence of Multidrug Resistant *Pseudomonas aeruginosa* (MDRPA) and Pandrug Resistant *Pseudomonas aeruginosa* (PDRPA) was 22% (54) and 4% (11) respectively. Their findings were based on the study of 245 patients.

According to the study Galdstone *et al.*, 2005, showed that *Pseudomonas aeruginosa* was highly resistant (42.8%) to carbapenem in patients with respiratory tract infections admitted in ICUs.

The resistance produced by *Pseudomonas aeruginosa* to various antimicrobial drugs is frequent in intensive care units and may be associated with the excess use of some specific drugs. The antibiotic resistance is a major concern for microbiologist. According to Rajat *et al.*, 2012 maximum isolates of *Pseudomonas aeruginosa* are resistant to tobramycin (68%) followed by gentamycin (63%), piperacillin (50%), ciprofloxacin (49%) and ceftazidime (43%) (Rajat *et al.*, 2012).

According to onq *et al.*, 2011 introduction of Meropenem is linked with the development of resistance in *Pseudomonas aeruginosa*. Excess use of carbapenem contributes to evolution of Gram-negative bacteria producing extended-spectrum β-lactamases, which increase antibiotic resistance in *Pseudomonas aeruginosa* (onq *et al.*, 2011).

1.10 Resistance towards antibiotics

Pseudomonas aeruginosa became resistant to several antimicrobials because of different structural classes. The resistance is either inherently or due to possession of genetic determinants. Most of the *Pseudomonas aeruginosa* isolates are defiant to ampicillin, amoxicillin-clavulanate, anti staphylococcal penicillins, narrow and extended-spectrum cephalosporins, tetracyclines, chloramphenicol, macrolides, and rifampin. *Pseudomonas aeruginosa* is also defiant to ampicillin-sulbactam and trimethoprimsulfamethoxazole. *Pseudomonas aeruginosa* may also develop antimicrobial resistance because of impermeability of outer membrane, amplified activity of multidrug efflux pumps, amendments in target site, or enzymatic mortification (*e.g.*, aminoglycoside-modifying enzymes and ß-lactamases).

Pseudomonas aeruginosa resistance towards noncarbapenem ß-lactams is related with excessive production of cephalosporinase (AmpC) (Kiska and Gilligan, 1999; Pollack, 2000)

The growing prevalence of *Pseudomonas aeruginosa* has gained interest in recent years. In developing countries prevalence of *Pseudomonas aeruginosa* in postoperative patients is much higher with severe complications because of poor hygienic measures, low quality antiseptic, and lack of proper medicinal treatment (Bertrand *et al.,* 2002).

1.11 Antibiotics Produced from *pseudomonas* Strains

Many isolates of this genus have been investigated globally for bioactive compounds. Many of the strains produce bioactive compounds, enzymes, and biosurfactants. Some *Pseudomonas* isolates are used for biological control of plants diseases and bioremediations. Few marine isolates have also been described to produce novel antibiotics.

Emmerich and Low isolated bacterium named *Bacillus pycyaneus* from infected bandage that caused green color infections in open wounds. Later this bacterium was named *Pseudomonas aeruginosa.* Blue green color of the infections is because of a green blue phenazine pigment called pyocyanin. They combined the isolates with other bacteria and found that it has the ability to demolish other strains of bacteria. The demolished bacteria were those that caused cholera, typhoid, diphtheria, and anthrax. Emmerich and Low isolated the extracts of *Bacillus pycyaneus* and named it *pyocyanase.* It was the first antibiotic to be used but unfortunately its activity was different in different patients, it had large amount of phanazaine such as pyocyanin which made it quite toxic to human as a result the drug was finally neglected (Emmerich *et al.,* 1899).

Cepacin A and cepacin B are two new antibiotics which have been secluded from the fermentation broth of *pseudomonas cepacia* SC 11,783. Cepacin A is active against *staphylococci* but has weak activity against *streptococci* and the majority of gram-negative organism. Cepacin B has exceptional activity against *staphylococci* and some Gram-negative organisms (Parker *et al.,* 1984).

There is abundant amount of studies on antibiotics producing *Pseudomonas* spp is because of the reason that pseudomonads are common residents of rhizosphere and phyllosphere, are easily isolated and from natural environment, utilize a wide range of substrates, easily to culture and manipulate genetically, making them more acquiescent to experimentation.(leisinger and Margraff, 1979; whipps, 2001).

Few important antibiotics produced by *pseudomonas* are 2, 4 diacetylphloroglucinol, pyoluteorin, Rhamnolipid 1, pyrrolnitrin, Phenazine 1-carboxilic acid, Amphisin.

1.12 2, 4 diacetylphloroglucinol (2, 4 DAPG)

Many bacteria, algae and plants produce phenolic compounds called phlroglucinol (Reddi and Borovokov, 1969; verrota *et al.*, 1999; Jimenez_Escig *et al.*, 2001). More than 60 derivatives of phlrogluicnol have been produced, out of which three are produced by *Pseudomonas* spp. It includes monoacetylphlroglucinol (MAPG), 2, 4 DAPG, and tri-acetylphlroglucinol (TAPG) (Reddi and Borovokov, 1969; Shanahan *et al.*, 1992).

These three compounds play an significant role in the area of biological control of plants diseases. Various studies have reported that 2, 4 DAPG can restrain a huge variety of plant pathogen including bacteria, fungi and nematodes (Stutz *et al.*, 1986; Keel *et al.*, 1990; Vincent *et al.*, 1991;Fenton *et al.*,1992; Levy *et al.*, 1992; Keel *et al.*, 1992; Cronin *et al.*, 1997a, b; Duffy and Defago, 1997; Sharifi-tehrani *et al.*, 1998)

2, 4 DAPG producing pseudomonas spp suppress the growth of fungi *Gaeumannomyces graminis* on wheat, *Thielaviopsis basicola* on tobacco, *Fusarium oxysporum f sp. Radicis-lycopersici* on tomato and the oomycete *pythium ultimum* on sugar beet and cucumber (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Stutz *et al.*, 1986; Duffy and Defago, 1997; Sharifi-tehrani *et al.*, 1998).
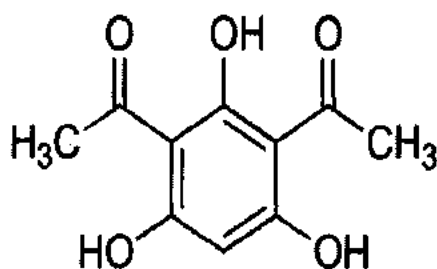
Figure: 1 Chemical Structure of 2, 4 Diacetylphloroglucinol (Souza, 2002)

It has been reported that 2, 4 DAPG induce resistence in plant's defense mechanism against pathogens. This hypothesis is based on the fact that herbicides, which acts as inducer of plant's defense mechanism are simillar in structure to 2, 4 DAPG (Altman and campbell,1977; cohen *et al.*, 1986).

Pseudomonas species producing DAPG have been found in the rhizospheres of different crops grown in soils from various geographic regions (Keel *et al.*, 1996), and they are predominant components of the wheat plant's rhizosphere, grown in soils that naturally repress take-all disease (Souza *et al.*, 2003; McSpadden *et al.*, 2000; Raaijmakers *et al.*, 1997; Weller *et al.*, 2002). They also have been found in the soils that naturally resistant to black root rot of tobacco (Keel *et al.*, 1996; Ramette *et al.*, 2003) or *Fusarium* wilt disease (Landa *et al.*, 2002). Recently, in the medical area, DAPG also has drawn attention because of its bacteriolytic activity against multidrug-resistant *Staphylococcus aureus* (Isnansetyo and Kamei, 2003).

1.13 Pyoluteorin (PLT)

PLT 4, 5 dichloro-1 H-pyrrol-2-y-2, 6-dihydroxyphenyl ketone is a phenolic polyketide antibiotic and its resorcinol ring is linked to bichlorinated pyrrol moiety Figure no.2.
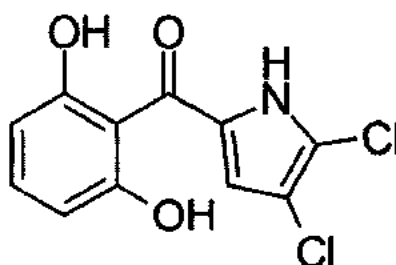
Figure no: 1.1chemical Structure of Pyoluteorin (Souza, 2002)

PLT was first isolated in *pseudomonas aeroginosa* (Takeda, 1958) and later it was found in *pseudomonas aeroginosa* strain S10B2 (Ohmori *et al.*, 1978) and *pseudomonas fluorescens* strain Pf-5 and CHAO (Bencini *et al.*, 1983; Bender *et al.*, 1999; Keel *et al.*, 1996).

PLT has bactericidal, herbicidal, fungicidal, and oomycidal activities, in particular against *pythium* spp but its precise mode of action is still not clear (Ohmori *et al.*, 1978; Takeda, 1958; Maurhoffer *et al.*, 1992). Pure PLT treated cotton seeds are resistant to *pythium ultimum*, causing damping-off (Howell and Stipanovic, 1980).

Derivatives of *pseudomonas* strains which were either defective in PLT production or produced higher amount of PLT, have indicated that role of PLT in biological control may be different among different plants hosts (Maurhoffer *et al.*, 1992, 1994, 1995; Kraus and Loper, 1992). PLT production in the rhizosphere by CHAO strain and plt gene expression in spermoshere by pf-5 strain is different among different host plants, most probably distressing the biocontrol activity of PLT (Maurhoffer *et al.*, 1995; Kraus and Loper, 1995).

1.14 Phenazines (PHZ)

Nitrogen containing phenazines compounds are exclusively produced by bacteria (Turner and Messenger, 1986). PHZs are diverse antibiotics have more than 50 derivatives. Several different strains produce common PHZs and several different PHZs are produced by common strains (Turner and Messenger, 1986). It was found that PHZs are toxic to fungi, bacteria, and algae (Toophey et al., 1965).

PHZs modes of action include cell death or injury due to their ability to endure redox cycling in presence of several reducing agents and molecular oxygen, which leads to accumulation of toxic oxygen species (Hasan and Fridovich., 1980). Some PHZs intercalate with GC base pairs of DNA strands and inhibit RNA synthesis (Turner and Messanger, 1986), and to disturb electron transport and energy production (Baron et al., 1989). PHZs play a key role in virulence in human and animals, biological control and competitiveness. *Pseudomonas aeruginosa* produce PHZs compound pyyocyanin is a virulence factor in the infection of cystic fibrosis patients (Wilson et al., 1987). Phenazine-1 carboxilic acid (PCA) produces by *Pseudomonas fluorescens* 2-79 and three PHZs compounds: PCA, 2-Hydroxy-phenazine-1 carboxilic acid, 2- hydroxyl-phenzine are produced by *pseudomonas aureofaciens*. These PHZs compounds were found to play a vital role in the competitive ability of vital strains in the rhizosphere (Mazzola et al., 1992).

PHZs are also important in biocontrol of several plants diseases. Those mutants which are poor in PHZ biosynthsis are unable to control *G. graminis* var. *trtici* and *Septoria trtici* on wheat (Thomashow and Weller, 1988; Flaishman et al., 1990; Pierson III ad Thomashow, 1992). *Fusarium oxysporum f.sp. ciceris* on chickpea and *pythium splendens* on beans and lettuce (Anjaiah et al.,1998) and *Fusarium oxysporum f.sp. radici-lycoperici* on tomato (Chin et al., 1998). Mutants genetically restored their PHZs production ability and thus become able to restrain fungal growth on artificial medium and to control the disease in plants (Thomashow and Weller, 1988; Pierson III ad Thomashow, 1992). The role of PHZs in the biocontrol was provided by studies with genes responsible for the expression of PHZ biosynthetic operon in the rhizosphere (Chin et al., 1998) and its recognition on roots of wheat treated with PHZ-producing strains (Thomashow et al., 1990).
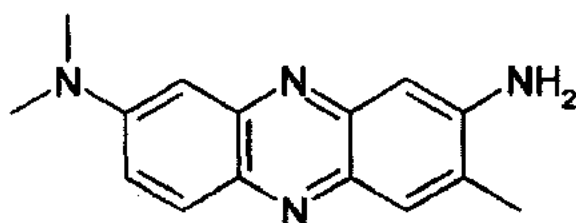
Figure: 1.2 chemical structure of PHZ (Souza, 2002)

1.15 Pyrrolnitrin (PRN)

PRN (3-chloro-4(2'-nitro-3'chlorophenyl)-pyrrol) is a chlorinated phenylpyrrol antibiotic. Its production is observed in many bacterial genera including, *pseudomonas, Burkholderia, enterobacter, Serratia,* and *Myxococcus* (Arima *et al.*, 1964; Elander *et al.*, 1968; Hammer *et al.*, 1999; Kalbe *et al.*, 1996).

PRN interfere with respiratory electron transport system (Tripathi and Gottlieb, 1964). PRN is an active compound against several bacteria and fungi, in particular *Rhizoctonia solani* (Arima *et al.*, 1964; Cartwright *et al.*, 1995; Chernin *et al.*, 1996; El-banna and Winkelmann, 1988; Hill *et al.*, 1994; Jayaswal *et al.*, 1993; Rosales *et al.*, 1995). PRN has also a role in control of post harvest diseases caused by *Botrytis cinerea* on apple, pear and on cut flowers (Hammer and Evensen, 1993; Janisiewiczand and Roitman, 1988), and it has been used against opportunistic fungi infections in humans (Tawara *et al.*, 1989). Mutants of *pseudomonas fluorescens* strain BL915 and Pf-5 are incapable to control *R. solani* on cotton *pyrenophora tritici-repentis* in axenically colonized wheat straw because they are defective in PRN production (Hill *et al.*, 1994; Pfender *et al.*, 1993). The antibiotic was also found on wounded potatoes where it was colonized by a *B.cepacia* strain (Burkhead *et al.*, 1994).
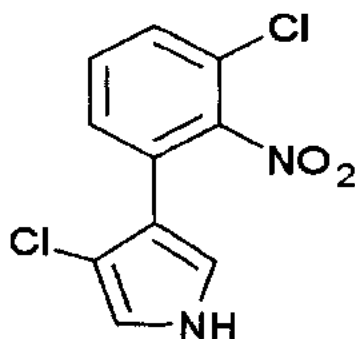
Figure: 1.3 chemical structure of Pyrrolnitrin (Souza, 2002)

1.16 Biosurfactants antibiotics

Many surfactants (surface-active molecules) have been described which are of biological origin (Feichter, 1992). These molecules have hydrophilic and hydrophobic regions in the same molecule that's why they have surfactant properties. There are numerous classes of biosurfactants which have different chemical structures and surface properties. Biosurfactants can be classified into two classes, low-molecular-weight polymers and high molecular weight polymer. Low molecular weight polymer low surface and interfacial tensions and high-molecular-weight polymers bind to surfaces (Ron and Rosenberg, 2001). *Pseudomonas* species produce several biosurfactants that have antibiotic activity in addition to their surfacetant activity. These surfactant molecules include rhamolipids and peptide biosurfactants and they are classified as low-molecular-weight polymers.

i.   **Rhamnolipids (RHLs)**

Rhamnolipids (RHLs) are glycolipid biosurfactants molecules containing rhamnose and β-hydroxydecanoic acid and are produced specifically by *Pseudomonas aeruginosa* and some other species of fluorescent Pseudomonads, (Itoh *et al.*, 1971; Van Dyjke *et al.*, 1993).There are six different types of RHLs. The difference is based on the rhamnose content and in the length and chemical nature of their lipid fraction (Jarvis and Johnson, 1949; Hirayama and Kato, 1982; Syldatk *et al.*, 1985). The biosynthesis, genetics, and regulation of RHL production in *Pseudomonas aeruginosa* are very well discussed in literature (Hauser and Karnovsky, 1957; Ochsner and Reiser, 1995). *Pseudomonas aeruginosa* use RHLs as virulence factors during colonization of the human lung tissue (Iglewski, 1989). RHLs also have antibiotic effects, acting in solubilization of cell envelopes of opposing microorganisms (Kurioka and Liu, 1967). In vitro RHLs also show antibacterial, mycoplasmacidal, cytotoxic, and antiviral activities (Jarvis and Johnson, 1949; Edwards and Hayashi, 1965; Hisatsuka *et al.*, 1971; Itoh *et al.*, 1971; Hirayama and Kato, 1982). Recently it is demonstrated that RHLs also have a role in the biocontrol of plant pathogens (Stanghellini and Miller, 1997; Kim *et al.*, 2000). RHLs were vigorous against zoospores of oomycetes, causing termination of motility and lysis of whole zoospore populations in less than 1 min. RHL-producing bacterium introduction into a re-circulating hydroponic system gave excellent control of *Phytophthora capsici* on pepper although this control is temporary (Stanghelliniand Miller, 1997). RHL B produced by *Pseudomonas aeruginosa* B5

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inhibited the *Pseudomonas capsici* mycelial growth and *Colletotrichum orbicularie* spore germination in vitro. These pathogen causing diseases in pepper and cucumber plants which were also suppressed when treated with purified RHL B (Kim *et al.*, 2000). RHLs are mode of action is thought to act by intercalating into the plasma membrane of zoospores and cause its distraction (Stanghellini and Miller, 1997). However, their exact mode of action on structures protected by cell walls has not been demonstrated.
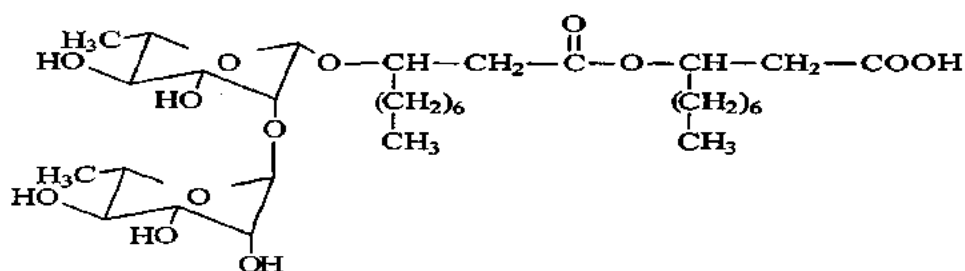
Figure: 1.4 chemical structure of Rhamnolipid (RHLs) (Souza, 2002)

ii. Lipopeptide

Lipopeptide surfactants have a peptide moiety associated with a fatty acid. The peptide moiety is synthesized non-ribosomally by a multi-enzyme peptide synthase complex (Ron and Rosenberg, 2001). Several lipopeptide surfactants, e.g. viscosin (Hiramoto *et al.*, 1970), viscosinamide (Nielsen *et al.*, 1999), massetolides (Gerard *et al.*, 1997), tensin (Nielsen *et al.*, 2000), syringopeptins, syringomycins, syringostatins, syringotoxins, and pseudomycins (Hutchison and Gross, 1997; Serra *et al.*, 1999), tolaasin (Rainey *et al.*, 1991), the white line inducing principle (WLIP) (Morthire *et al.*, 1991), pholipeptin (Ui *et al.*, 1997), arthrofactin (Morikawa *et al.*, 1993), amphisin (Sorensen *et al.*, 2001) and ecomycins (Miller *et al.*, 1998) are produced by *Pseudomonas* species. Lipopeptides produced by *pseudomonas* are tested to have antimicrobial properties. *Pseudomonas syringaeare* produce lipopeptides syringopeptin, syringostatins, syringomycins, syringotoxins, and pseudomycins are virulence factors in the diseases caused by these phytopathogenic bacteria (Scholz *et al.*, 2001). Syringomycins, syringostatins, syringotoxins, and pseudomycins also have antifungal activity and are very much helpful for the development of agents to control plant and human diseases, here as compared to other syringopeptins have stronger phytotoxic activity then antifungal activity (Lam *et al.*, 1987;

Iacobellis *et al.*, 1992). *Pseudomonas tolaasii* produce tolaasin which is the primary bacterial compound accountable for developing disease symptoms on cultivated mushrooms (Godfrey *et al.*, 2001) and is also active against a range of basidiomycetes and Gram-positive bacteria (Rainey *et al.*, 1991). WLIP produced by an opportunistic pathogen *Pseudomonas reactans*, correlated to *Pseudomonas tolaasii*, was proposed to act as an inhibitor of the symptoms elicit by *Pseudomonas tolaasi* on mushrooms (Soler *et al.*, 1999). Viscosin, a lipopeptide have antifungal and surfactant properties, is an significant virulence factor in the disease provoked by

Pectolytic strains of *Pseudomonas fluorescens* on brocoli (Hildebrand *et al.*, 1998). For the control of pathogenic fungi, tolaasin and viscosin are also proposed to be used as biological agents (Nielsen *et al.*, 1999). *Pseudomonas viridiflava* produce ecomycins were reported to have an active role against numerous human and plant pathogenic fungi (Miller *et al.*, 1998).

Pseudomonas fluorescens strains residing in soil produce viscosinamide and tensin which were reported to have role in the inhibition of mycelial growth of several fungi and oomycetes, inducing hyphal swellings and stimulating hyphal branching (Thrane *et al.*, 1999; Nielsen *et al.*, 2000). Several cyclic depsipeptides called massetolides are produced *pseudomonas* species from marine tube worms (Gerard *et al.*, 1997). Massetolides are correlated to other lipopeptide surfactants, such as viscosinamide and viscosin. Massetolides showed distinct activity against *Mycobacterium* species. *Pseudomonas fluorescens* produced amphisin and pholipetin related compounds (Ui *et al.*, 1997; Sorensen *et al.*, 2001), and a *Pseudomonas* species produced arthrofactin (Morikawa *et al.*, 1993). Biological activity of these compounds is not yet investigated, but because their structures are similar to other lipopeptides so similar activities might be expected.

The mode of action of numerous lipopeptide surfactants involves formation of ion channels in the cell wall and disruption in the cell membrane structure (Nielsen *et al.*, 1999; Hutchison and Johnstone, 1993; Vanittanakom and Loeffler, 1986; Thrane *et al.*, 1999).

One of the major health problem faced by the world is the emergence of multidrug-resistant bacteria (Buke *et al.*, 2007; Strateva *et al.*, 2007). Among these bacteria *Staphylococcus aureus* is one of the most important human pathogens. It is linked with hospital and community acquired infections. In different countries the number and proportion of *S. aureus* (MRSA) is increasing over the last few decades (Stefani and Varaldo, 2003; Rodríguez *et al.*, 2010; Lee *et al.*, 2011) and other animals, such as pigs, cats, dogs, cattle and exotic species (Smith and Pearson, 2011;

Weese, 2010). According to the data of the SENTRY Antimicrobial Surveillance Program, MRSA strains were present in the list of most prevalent pathogens and caused 56% of the nosocomial and community infections in Brazil (Sader *et al.*, 2004). MRSA Brazilian epidemic clone (BEC) is one of the major global clones, a hospital-acquired MRSA strain. Its isolates are usually resistant to multiple antimicrobials (Oliveira *et al.*, 2001).

1.17 Methicillin Resistant *Staphylococcus aureus* (MRSA)

The health hazards related with MRSA infections, including its ability to produce persistent infections, predominantly in susceptible patients, and its resistance to multiple antibiotics, is a warranting alarm for the monitoring programs to control its dissemination. Considerable efforts are being done regarding its epidemiology to find out its strains in the population and the dynamics of clonal spread (Crisostomo *et al.*, 2001). MRSA infections were treated with Vancomycinm for many years and it has been considered as a drug of choice against MRSA infections however, after the appearance of vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) the antimicrobial therapy has become more difficult. Therefore, in order to control and treat infections caused by these microorganisms, many new chemotherapeutic compounds have been studied and developed (O'Neill, 2008).

In recent years some natural antibacterial agents such as, *Hylomecon hylomeconoides, Quercus dilatata Eleutherine Americana, Chelidonium majus Linn. (Papaveraceae) and Tabebuia avellanedae* compounds have been studied against MRSA. (Jamil *et al.*, 2012; Choi *et al.*, 2010; Ifesan *et al.*, 2009; Zuo *et al.*, 2008; Pereira *et al.*, 2006). Some of the important antibacterial compounds produce by other bacteria have been tested against methicillin-sensitive *S. aureus* (MSSA) and MRSA (Hashizume *et al.*, 2008; Cazoto *et al.*, 2011; Ding *et al.*, 2011).

In our study we molecularly characterized medically important *pseudomonas* strains and also checked out their activity against MRSA and environmental bacteria.
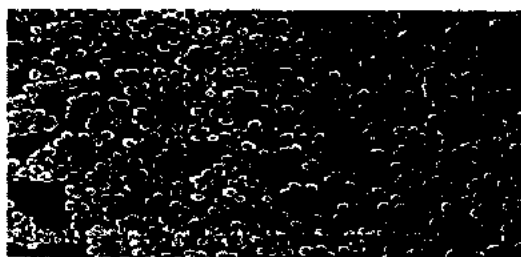
Figure: 1.5 electron microscopic views of MRSA
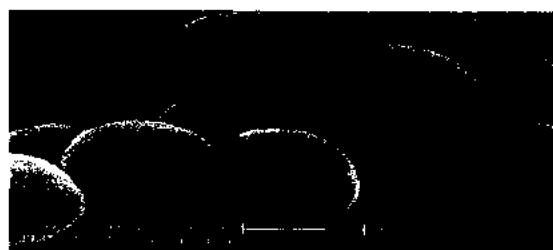
Figure: 1.6 electron microscopic views of VRSA

1.18 AIMS AND OBJETIVES

The present study aims to

1. Isolation of *Pseudomonas* strains from clinical specimens
2. Screening of the isolated strains for the production of antimicrobial compounds
3. Determination of antibiotics resistance pattern
4. Molecular identification of the antimicrobial compounds producing strains

MATERIALS AND METHODS

2. Materials and Methods

The present study was conducted in Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad and institute of biomedical and genetic engineering (IBGE), KRL hospital Islamabad to identify and molecularly characterize *Pseudomonas* strains isolated from clinical samples, screen these strains for the antimicrobial compounds production and to find out antibiotics resistance pattern of these strains. The details of the materials used in this study are given bellow:

2.1 Sterilization of Apparatus

All the glass ware used in this study was sterilized by subjecting them to high pressure 15psi saturated steam at 121°C for around 15-20 minutes according to the procedure given by (Harrigan, 1998).

2.2 Growth Media

Nutrient Agar, Nutrient broth, Trypton Agar and Trypton broth, MacConkey agar, blood agar and CLED agar of Merck Company were used for the cultivation and isolation of bacterial strains in this study.

2.3 Selected Microorganisms

The test organisms, such as *Pseudomonas* strains and methicillin-resistant *Staphylococcus aureus* (MRSA), were obtained from pathological laboratory of KRL hospital, Pakistan Institute of Medical Sciences Islamabad (PIMS) and Islamabad Diagnostic Center (IDC) and maintained on nutrient agar and trypton agar for use in bioassay. Microorganism was aseptically transferred into sterile trypton broth from test microorganisms and incubated for 24 hours at 37°C.

2.4 Sample Collection

For conducting this study, fifty clinical samples, three MRSA samples and two environmental bacteria samples had been collected. The origin of the samples was pus, blood, urine and wound swabs of patients. All the three MRSA samples were obtained from wounds pus of different patients. Two environmental bacteria were obtained from natural sources i.e. air. All the *Pseudomonas* samples were tested for antimicrobial production against the selected MRSA and

environmental bacteria. Those *Pseudomonas* strains which produced ideal zones of inhibition against the selected microorganisms were further molecularly characterized through 16S rRNA gene sequencing. Specific antibiotics were tested against all the antimicrobial compounds producing *Pseudomonas* strains and antibiotics resistance pattern of these strains and selected MRSA strains was determined.

2.5 Maintenance of the Culture

The pure cultures were maintained at refrigeration temperature and sub-culturing on nutrient agar and trypton agar after every ten days in order to prevent contamination. Pure cultures were also grown in test tubes containing sterile trypton broth, which were used in the DNA extraction protocol.

2.6 Morphological Characteristics

Colonial morphology of the microorganisms was examined on solid medium. On nutrient agar *Pseudomonas* strains produced circular dark green, light green, yellowish green and colorless colonies. The two environmental isolates appeared as orange and yellow colored while MRSA produced colorless colonies. Cellular morphology of the isolated strains was examined by Gram's staining.

2.7 Bichemical Tests

The cultures were identified on the basis of biochemical tests as recommended by (Harrigan, 1998)

i.   **Catalase Test**

The catalase test was conducted in order to find out catalase production ability of the bacteria. This test was performed by pouring 1ml of 3% freshly prepared hydrogen peroxide on an agar culture. The catalase activity was confirmed through production of air bubbles. Catalse test is marked as positive if bubble production occurred.

ii.   Oxidase Test

This test is used to detect the presence of cytochrome oxidase that will catalyze the transport of electrons between electron donors and redox dye. In the reagent Tetramethyl-p-phenylene diamine dihydrochloride diminished its color to deep purple. Oxidase reagent contains 10g/l or 1% solution of tetramethyl-p-phenylene diamine dihydrochloride. To perform this test a piece of filter paper was placed in petri dish and 3 drops of oxidase reagent was added. With the help of sterile glass rod, a colony of test organisms was removed from a culture plate and smeared it on the filter paper. Within 5-10 seconds bacteria gave blue color in oxidase positive, while no such change occurs in oxidase negative bacteria.

iii.   TSI Agar Test

Triple sugar iron agar (TSI) test is used to distinguish bacteria on their sulfur reduction and carbohydrates fermenting ability. TSI agar contains sucrose, lactose, a small amount of glucose, ferrous sulfate, and phenol red for pH indication.

2.8 Screening of *Pseudomonas* Strains for Antibiotics Productions

All *pseudomonas* strains were screened for antibiotics production by Burkholder Agar Diffusion Assay (Burkholder *et al.*, 1966) and Chloroform Overlay Assay (Uzair, 2007).

I.   Agar Diffusion Method

Pseudomonas strains were grown in brain heart infusion broth and incubated at 30°C for 48 hours. After 48 hours cell free supernatants were obtained by centrifugation at 10, 000 rpm for 3 min at 4°C. The cells were removed and pH of the supernatant was adjusted to 7.0 with NaOH. A lawn of suspected sensitive strain was prepared on nutrient plates and wells of (4mm diameter) were cut and 100µl of supernatant of test organisms were poured into each well and plates were incubated at 30°C for 24-48 hours and zones of inhibition were examined.

II.   Chloroform Overlay Assay

Nutrient agar agar medium was prepared and poured into petri dishes under aseptic conditions in a laminar air flow. With the help of sterile wire loop *Pseudomonas* strains were dotted in the center of the solidified agar plates. Plates were incubated at 37°C for 24 hours. After 24 hours

Pseudomonas colony was removed from the nutrient agar plates with the help of sterile cotton bud. Plates were covered with filter paper having chloroform for 30 minutes. The bacteria which were to be tested against antibiotics produced by *Pseudomonas* were streaked on the plates with the help of sterile cotton bud. Plates were incubated at 37°C for 24 hours. Zones of inhibitions in mm were measured and noted.

2.9 Selection of Bacteria from Collected Samples

Out of forty *Pseudomonas* strains, thirty produced zones of inhibition against MRSA and environmental isolates. These thirty samples were re-cultured and further from them, those strains with maximum activity against MRSA and environmental isolates were molecularly characterized.

2.10 Antibiotics Resistance Pattern

Antibiotics resistance pattern of antibiotics producing *Pseudomonas* strains was determined by using sixteen different antibiotics discs. Antibiotics which were tested against *Pseudomonas* strains are as follow: Imipenem(10μg), Levofloxacin(5μg), Amikacin(30μg), sulbactam-Cefoperazone(105μg), Piperacillin(100μg), Ceftazidime(30μg), Tazobactam(110μg), Ciprofloxacin(5μg), Cefepime(30μg), Tobramycin(10μg), Cefotaxime(30μg), Meropenem(10μg), Amoxicillin(30μg), Ceftriaxone(30μg), Gentamicin(10μg), Polymixin B(300μg). in order to find out antibiotics resistance pattern a lawn of bacterial colony from the pure culture was made on the nutrient agar plate with the help of a sterile cotton bud. Antibiotic discs were placed on plate at a specific distance. Plates were then incubated at 37°C for 24 hours. After 24 hours plates were checked out for the zones of inhibitions. Antibiotics resistance pattern of the three selected MRSA samples were also determined by using antibiotics discs of DA(2μg), AZM(15μg), STX(25μg), Ciprofloxacin(5μg), C(30μg), VA(30μg), Tobramycin(10μg), FOX(30μg), AMP(10μg), Penicillin, CLR(15μg), TCP, MH(30μg), LZP(30μg), OXC and OFI.

2.11 Molecular Characterization of Isolated *Pseudomonas* Strains

For molecular characterization, genomic DNA was extracted by the following protocol.

i.   Genomic DNA Isolation From *Pseudomonas* Species

This protocol is used to isolate genomic DNA (gDNA) from *Pseudomonas* spp. (and any gram negative bacteria) is adapted from the Chen and Kuo, 1993 publication in nucleic acids research. This protocol has the following steps: A single bacterial colony was inoculated into 5ml of trptone broth and incubated at 37°C for 24 hours. Saturated culture of 1.5mL centrifuged for 3 min at 12000 rpm. Supernatant was discarded, and cell pellet was resuspended in 200uL of lysis buffer and mixed by intense pipetting. In order to get rid of proteins and cell debris, 66uL 5M NaCl was added and mixed well. Centrifugation was done at 12000rpm for 10 min at 4°C. Clear supernatant was transferred into another vial and chloroform of equal volume was added. Mixed by gently inverting tubes at least 50 times or until a formation of milky solution and then centrifuged for 3 min at 12000rpm. Supernatant was transferred into new vial and DNA was precipitated by adding 100% ethanol, and incubated at -20°C for at least 1 hour. After that centrifugation was done for 10 minutes at 4°C. Pellet was washed twice with 70% ethanol after the removal of supernatant. Pellet was dried in a speed vac or over a clean piece of paper towel for 30 minutes. The pellet was re-suspended in 50uL of d H_2O or TE buffer. DNA concentration was measured by using nanodrop.

ii.   DNA Extraction from Gram Positive Bacteria

The protocol which was adapted for the Gram positive MRSA and two environmental isolates has the following steps: A single bacterial colony was transferred into 5ml sterile trptone broth and incubated at 37°C for 24 hours. 1 ml suspension was centrifuged at 8000rpm for 5 minutes. Supernatant was discarded and cells were washed with 400 µl STE buffer and centrifuged at 8000 rpm for 5 minutes. This step was repeated twice. Each cell pellets were re-suspended in 200 µl TE buffer. 100 µl Tris-saturated phenol (pH 8.0) was added and vortex for 60 seconds to lyse cells and centrifuged at 13000 rpm for 5 minutes at 4°C to separate the aqueous phase from organic phase. Upper aqueous phase (160 µl) was transferred into a clean 1.5 ml tube. 40 µl TE buffer was added to make the volume up to 200 µl and then 100µl chloroform was added and centrifuged for 5 minutes at 13000rpm at 4°C. Chloroform extraction was repeated 2-3 times until white interface appeared. Upper aqueous phase 150 µl was transferred into a clean 1.5ml tube and was directly used for PCR.

2.12 PCR Amplification

PCR was performed in a reaction volume of 20 μl consisting of 10X PCR buffer (2 μl), 50mM MgCl$_2$ (0.6μl), dNTP mix (1.6μl), (0.6μl) of 16S-R and 16S-F primers, *taq* polymerase (0.4 μl), DNA (1 μl). dH$_2$O was added to attain the final volume. In negative control 5μl of dH$_2$O was added instead of DNA template in reaction mixture. Three different PCR programs were used for the amplification of 16S rRNA gene. First it was started according to the following program: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min., annealing at 55°C for 1 min, extension at 72°C for 1 min; and a final extension at 72°C for 7 minutes. The annealing temperature was gradually increased from 55°C to 65°C in order to get the ideal amplification of the gene.

Second condition used for the amplification was as follow: initial denaturation at 96°C for 10 min; 35 cycles of denaturation at 96°C for 10 sec., annealing at 59°C for 20 sec, extension at 72°C for 30 sec; and a final extension at 72°C for 10 minutes.

Third condition was as follow: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 45 sec., annealing at 59°C for 45 sec, extension at 72°C for 45 sec; and a final extension at 72°C for 10 minutes.

2.13 Agarose Gel Electrophoresis

The process of electrophoresis was carried out in 1.5% agarose gels containing ethidium bromide. Size of amplicons was determined by comparing it with bands of DNA ladder of 100 bp plus (fermentas). Gel image was saved and DNA bands of the desired size were cut from the gel blocks.

2.14 DNA Bands Extraction from Agarose Gel

Specific DNA bands were cut from gel because there was some non-specific bands also appeared in the agarose gel. The protocol has the following steps:

Agarose gel containing DNA fragment was excised with the help of a clean, sharp scalpel. Extra agarose was removed in order to minimize the gel slice. Weight of the gel slice was measured in a colorless eppendorf tube and 3 volumes of QG buffer to the volume of gel were added. Eppendorf tubes containing gel was incubated at 50°C for 10 minutes or until the gel had

completely melted. During incubation tubes were vortexed after every 2-3 minutes. When the color of the mixture was as yellow as the buffer QG without dissolved agarose, isopropanol equal to 1 gel volume was added to the sample and mixed well. QIAquick spin column was placed in 2 ml collection tube. For binding the DNA, sample was applied to the QIAquick column and centrifuged for 1 minute. The flow through was removed and column was placed again in the same collection tube to re-use. 0.5ml QG buffer was added to QIAquick column and centrifuged for 1 minute. The flow was removed again and QIAquick column was centrifuged for one more minute at 13000 rpm. QIAquick column was placed in a clean 1.5ml micro-centrifuged tube. For the elution of DNA 50 µl buffer EB or water was added to the center of the QIAquick membrane and centrifuged the column for 1 minute at maximum speed. Alternatively 30 µl elution buffer was added to the center of the QIAquick membrane and let it stand for 1 minute and then centrifuged again for 1 minute. QIAquick column was discarded and DNA was collected in micro-centrifuged tubes.

2.15 Purification of PCR Amplicons

In order to remove primers and dNTPs PCR product was purified by precipitation method. To 15 µl of PCR product 1.5 µl of 10mM ammonium acetate and 30µl of absolute ethanol was added. It was then left on ice for 20 minutes. After centrifugation at 13000rpm for 10 minutes at room temperature, the supernatant was discarded and pallet was washed with 100 µl of 70% ethanol. After centrifugation at high speed the supernatant was again discarded and pallet was vacuum dried for one hour in desiccators at 40 °C and re-suspended in 20 µl of deionized water. The purified product was further amplified by using single primer through sequencing PCR.

2.16 Identification by 16S rRNA Gene Sequencing

Among the thirty antibiotics producing *pseudomonas* strains, six were selected for sequencing. Selection was done on the basis of zone of inhibition produced by them and pigments production. Two environmental isolates were also selected for 16S RNA gene sequencing. A 1200 bp fragment of the 16S rRNA gene of the bacterial strains was amplified using the universal primer pair F: GAGTTTGATCCTGGCTCAG and R: AGAAAGGAGGTATCCAGCC. For sequencing reaction each reaction volume has 5 µl DNA,

1 µl primer either forward or reverse and 4 µl bigdye. PCR conditions for sequencing reaction were as follow: 25 cycles of 96°C for 1 min., 96°C for 15 sec, and 60°C for 4 minutes.

2.17 Purification of the Sequencing Reaction Product

Sequenced PCR product was again purified to get rid of dye-dNTPs. To each 10 µl sequencing reaction product 2.5 µl of 125 mM EDTA and 30µl absolute ethanol was added, vortex mixed and left on ice for 15-20 minutes. Samples were then centrifuged at 14000rpm for 20 minutes. Supernatant was removed and 30µl 70% ethanol was added to each tube. Pellet was re-suspended with the help of pulse vortex for 1-2 minutes and again centrifuged at 14000rpm for 10 minutes. Supernatant was discarded and tubes were vacuum dried. Pellet was re-suspended in 10 µl of Hidi formamide. Then 10 µl of each sample was loaded in ABI plate and denatured at 95°C for 3 minutes. Immediately samples were placed on ice for at least 5 minutes and placed in ABI 3130 Genetic analyzer.

The consensus sequences were then used to compare with online databases (NCBI BLAST — http://blast.ncbi.nlm.nih.gov/Blast.cgi)

RESULTS

3. Results

In the present study a total of fifty clinical samples were collected for the isolation of *Pseudomonas* strains. Samples were screened for the presence of *Pseudomonas* strains. Identification of *Pseudomonas* strains was done on the basis of morphological characteristics of bacteria and biochemical tests such as Gram's staining, catalase, oxidase tests and TSI agar test. Detail morphological characteristics and results of biochemical tests are described in table no 3 and table no 3.1 respectively. Out of fifty samples forty samples were declared as *Pseudomonas* strains. Out of forty *Pseudomonas* strains 23 (57%) were isolated from pus, 8 (20%) from blood, 5 (12%) from urine and 4 (10%) from wounds swabs. Percentage of the origin of *Pseudomonas* strains are mentioned in (figure no 3).

3.1 Morphological Characteristics

Colony morphology of fifty samples grown on nutrient agar was shown in table no 3. Among these fifty strains eight were dark green pigmented strains, thirteen were light green pigmented strains, four were yellowish green pigmented strains, six orange color pigmented strains and 10 were non-pigmented strains. Cellular morphology of fifty samples was shown in (table 3). Out of fifty clinical samples, forty three samples were Gram negative and seven samples were Gram positive.

3.2 Biochemical Testing

The biochemical tests results of all fifty samples were shown in table (table 3). The catalase results had shown that out of fifty samples forty samples were catalase positive and had the ability to hydrolyze hydrogen peroxide whereas ten samples were catalase negative and could not hydrolyze hydrogen peroxide. The oxidase test result had shown that out of fifty samples forty were oxidase positive and ten were oxidase negative. TSI agar test was applied on forty three Gram negative specimens and the results showed that in forty samples no fermentation occurred, the slant and butt remained red and it was not accompanied by H_2S and gas production. Two samples were only glucose fermenter and showed red slant and yellow butt accompanied by H_2S and gas production. Yellow slant, yellow butt accompanied by H_2S and gas production was observed in one sample which means that this bacterium was glucose, sucrose and lactose fermenter.

3.3 Screening of *Pseudomonas* Strains for the Production of Antimicrobial Compounds

All of the forty *Pseudomonas* strains were screened for the antimicrobial compounds producing ability. Their activity was checked against three methicillin resistant *Staphylococcus aureus* (MRSA) and two environmental isolates. Two different methods were used to screen out their antimicrobial compounds producing ability.

i. Agar Well Diffusion Method

Out of forty *Pseudomonas* strains twenty six produced zones of inhibition against MRSA-1, fifteen against MRSA-2, nine against MRSA-3, seventeen against yellow environmental bacteria and twenty four against orange colored environmental bacteria. Results and details about the zones of inhibitions are described in table 3.2.

ii. Chloroform Overlay Assay

The strains which showed positive activity in agar well diffusion method were also active in chloroform overlay assay. There was a little difference in zones of inhibition of bacteria. Results and detail about the zones of inhibitions are described in table 3.3.

3.4 Antibiotics Resistance Profiling

Pseudomonas strains showed highest resistance towards Ciprofloxacin($5\mu g$) 62% and 60% resistance to cefotaxime($30\mu g$), 40% resistance to imipenem($10\mu g$), 38% resistance to piperacillin($100\mu g$), 37% resistance to ceftazidime($30\mu g$) and gentamicin($10\mu g$), 53% resistance to tobramycin($10\mu g$), 33% resistance to sulbactam-cefoperazone($105\mu g$), ceftriaxone($30\mu g$) and levofloxacin ($5\mu g$), 30% resistance to meropenem($10\mu g$), 27% resistance to cefepime($30\mu g$), 13% resistance to tazobactam($110\mu g$) and amikacin($30\mu g$) (Figure no 3.1).

Polymixin B ($300\mu g$) was 100% active against all thirty isolates. They showed 48% sensitivity towards Ciprofloxacin($5\mu g$), 40% sensitive to cefotaxime($30\mu g$), 60% sensitive to imipenem($10\mu g$) and piperacillin($100\mu g$), 63% sensitive to ceftazidime($30\mu g$) and gentamicin($10\mu g$), 47% sensitive to tobramycin($10\mu g$), 67% sensitive to sulbactam-cefoperazone($105\mu g$), ceftriaxone($30\mu g$) and levofloxacin($5\mu g$), 70% sensitive to

meropenem(10µg), 73% sensitive to cefepime(30µg), 87% sensitive to tazobactam(110µg) and amikacin(30µg) (Figure no3.2).

Table 3 colonial morphology of collected samples

S. No	Sample id	Pigment	Shape	Margin	Elevation	Texture
1	P-1	Dark green	Circular	Undulate	Convex	Smooth shinny
2	P-2	Dark green	Circular	Undulate	Convex	Smooth shinny
3	P-3	Non-pigmented	Circular	Entire	Convex	Smooth shinny
4	P-4	Light green	Circular	Entire	Convex	Smooth shinny
5	P-5	Light green	Circular	Entire	Convex	Smooth shinny
6	P-6	Dark green	Circular	Entire	Convex	Smooth shinny
7	P-7	Non-pigmented	Circular	Entire	Convex	Smooth shinny
8	P-8	Yellowish green	Circular	Entire	Convex	Smooth shinny
9	P-9	Light green	Circular	Entire	Convex	Smooth shinny
10	P-10	Light green	Circular	Entire	Convex	Smooth shinny
11	P-11	Light green	Circular	Entire	Convex	Smooth shinny
12	P-12	Non-pigmented	Circular	Entire	Convex	Smooth shinny
13	P-13	Light green	Circular	Entire	Convex	Smooth shinny
14	P-14	Light green	Circular	Entire	Convex	Smooth shinny
15	P-15	Light green	Circular	Entire	Convex	Smooth shinny
16	P-16	Light yellow	Irregular	Entire	Convex	Rough
17	P-17	Off white	Circular	Curved	Raised	Dry
18	P-18	Light green	Spindle	Entire	Raised	Smooth shinny
19	P-19	Orange	Circular	filamentous	Convex	Smooth shinny
20	P-20	Light green	Circular	Entire	Convex	Rough
21	P-21	Non-pigmented	Irregular	Entire	Convex	Smooth shinny
22	P-22	Light green	Circular	Entire	Convex	Rough
23	P-23	Off white	Circular	Entire	Convex	Dry

24	P-24	Off white	Circular	Entire	Convex	Smooth shinny
25	P-25	Off white	Spindle	Curved	Flat	Smooth shinny
26	P-25299	Light green	Circular	Entire	Convex	Smooth shinny
27	P-1419	Light green	Circular	Entire	Convex	Smooth shinny
28	P-24357	Dark green	Circular	Entire	Convex	Smooth shinny
29	P-1207	Light green	Circular	Entire	Convex	Smooth shinny
30	P-1086	Light green	Circular	Entire	Convex	Smooth shinny
31	P-1843	Colorless	Circular	Entire	Convex	Smooth shinny
32	P-25167	Yellowish green	Circular	Entire	Convex	Smooth shinny
33	P-298	Dark green	Circular	Entire	Convex	Smooth shinny
34	P-21713	Yellowish green	Circular	Entire	Convex	Smooth shinny
35	P-23612	Yellowish green	Circular	Entire	Convex	Smooth shinny
36	P-21361	Dark green	Circular	Entire	Convex	Smooth shinny
37	P-22502	colorless	Circular	Entire	Convex	Smooth shinny
38	P-23498	Dark green	Circular	Entire	Convex	Smooth shinny
39	P-1420	Light green	Circular	Entire	Convex	Smooth shinny
40	P-547	Dark green	Circular	Entire	Convex	Smooth shinny
41	P-21347	Non-pigmented	Circular	Entire	Convex	Smooth shinny
42	P-24244	Light green	Circular	Entire	Convex	Smooth shinny
43	P-23927	Non-pigmented	Circular	Entire	Convex	Smooth shinny
44	P-23811	Dark green	Circular	Entire	Convex	Dry
45	P-45	Light yellow	Circular	Filamentous	Raised	Dry
46	P-46	Off white	Irregular	Entire	Flat	Smooth shinny
47	P-47	Off white	Irregular	Entire	Convex	Smooth shinny
48	P-48	Orange	Circular	Entire	Raised	Rough
49	P-49	Light yellow	Circular	Entire	Raised	Rough
50	P-50	Orange	Irregular	Entire	Convex	Rough
51	O.B	Orange	Circular	Entire	Convex	Smooth shinny
52	Y.B	Yellow	Circular	Entire	Convex	Smooth shinny

Table 3.1 Biochemical Tests of Collected Samples

S. N O	Sample id	Origin of sample	Gram 's Stain	Catalase	Oxidase	TSI			
						BUTT	SLANT	GAS	H₂S
1	P-1	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
2	P-2	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
3	P-3	Urine	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
4	P-4	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
5	P-5	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
6	P-6	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
7	P-7	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
8	P-8	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
9	P-9	Urine	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
10	P-10	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
11	P-11	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
12	P-12	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
13	P-13	urine	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
14	P-14	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
15	P-15	Pus	-ve	-ve	-ve	Alkaline	Alkaline	-ve	-ve
16	P-16	Urine	+ve	-ve	-ve	-	-	-	-
17	P-17	Urine	+ve	-ve	-ve	-	-	-	-
18	P-18	Urine	+ve	-ve	-ve	-	-	-	-
19	P-19	Pus	-ve	+ve	-ve	Acid	Acid	+ve	+ve
20	P-20	Pus	-ve	+ve	-ve	-	-	-	-
21	P-21	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
22	P-22	Blood	-ve	+ve	+ve	Alkaline	Alkaline		
23	P-23	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
24	P-24	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
25	P-25	Pus	-ve	+ve	-ve	Acid	Alkaline	+ve	+ve
26	P-25299	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve

27	P-1419	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
28	P-24357	Wound swabs	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
29	P-1207	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
30	P-1086	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
31	P-1843	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
32	P-25167	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
33	P-298	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
34	P-21713	Urine	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
35	P-23612	Wound swabs	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
36	P-21361	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
37	P-22502	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
38	P-23498	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
39	P-1420	Blood	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
40	P-547	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
41	P-21347	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
42	P-24244	Urine	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
43	P-23927	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
44	P-23811	Wound swabs	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
45	P-45	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
46	P-46	Pus	-ve	+ve	+ve	Alkaline	Alkaline	-ve	-ve
47	P-47	Pus	+ve	-ve	-ve	-	-	-	-
48	P-48	Blood	+ve	-ve	-ve	-	-	-	-
49	P-49	Pus	-ve	+ve	-ve	Acid	Alkaline	+ve	+ve
50	P-50	Pus	+ve	-ve	-ve	-	-	-	-
51	O.B	Air	+ve	-ve	-ve	-	-	-	-
52	Y.B	Air	+ve	-ve	-ve	-	-	-	-

Figure no 3 Percentage of *Pseudomonas* specimens

%ge of recoverd *Pseudomonas* specimens from clinical strains
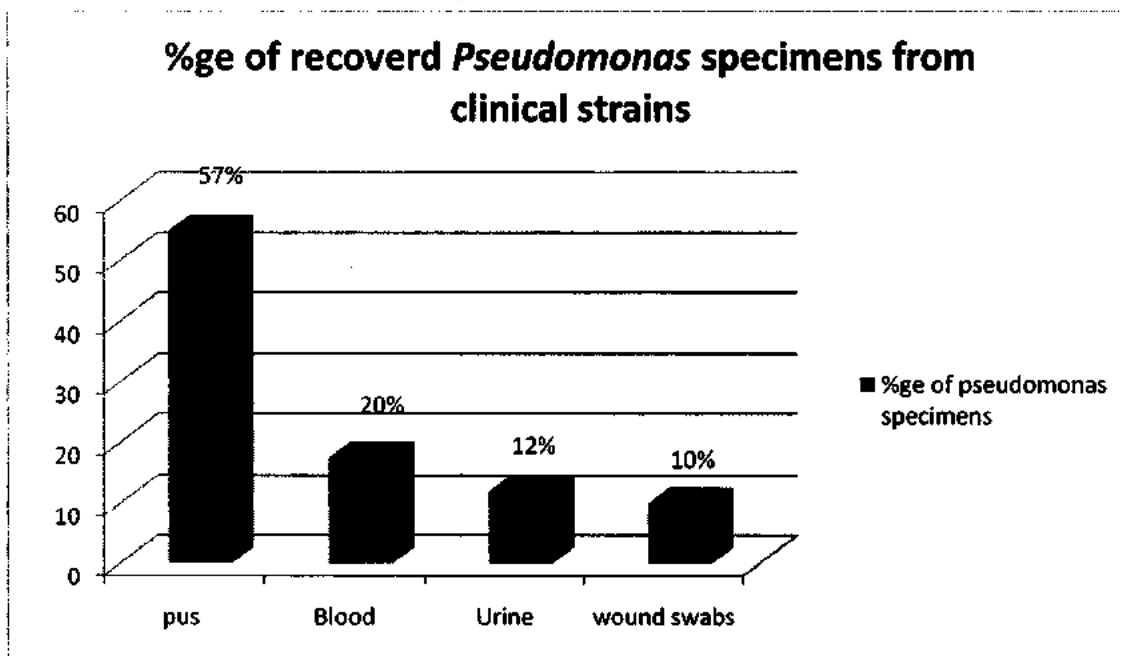
Table 3.2 Screening of *Pseudomonas* specimens for antimicrobial compound (Agar Well Diffusion Method)

S. No	Sample Code	MRSA Zone of Inhibition (mm)			Environmental Isolates	
		1	2	3	Y.B	O.B
1	P-1	32	20	_	20	25
2	P-2	_	_	_	25	_
3	P-3	35	25	_	_	20
4	P-4	30	25	_	15	30
5	P-5	50	25	_	35	40
6	P-6	40	35	25	30	29
7	P-7	45	_	_	41	60
8	P-8	_	_	_	44	45
9	P-9	40	25	_	40	20

10	P-10	40	35	–	–	–
11	P-11	–	30	–	45	40
12	P-12	30	–	–	24	20
13	P-13	–	–	–	–	–
14	P-14	25	26	–	33	–
15	P-15	37	34	20	41	44
16	P-21	–	–	–	–	–
17	P-22	–	–	–	–	–
18	P-23	–	–	–	–	–
19	P-24	–	–	–	–	–
20	P-25299	43	22	–	35	33
21	P-1419	40	–	23	–	–
22	P-24357	41	25	–	34	45
23	P-24244	–	–	–	–	–
24	P-1207	40	–	32		15
25	P-1086	35	–	–	–	44
26	P-1843	50	25	15	–	20
27	P-25167	30	23	–	–	20
28	P-298	48	–	–	38	35
29	P-21713	25	–	17	–	35
30	P-23612	37	35	25	20	26
31	P-23811	–	–	–	–	–
32	P-21361	40	–	–	–	45
33	P-22502	55	–	–	–	
34	P-23498	27	–	31	–	43
35	P-21347	–	–	–	–	–

36	P-1420	25	–	–	–	55
37	P-547	32	–	31	37	35
38	P-23927	–	–	–	–	–
39	P-45	–	–	–	–	–
40	P-46	–	–	–	–	–

Table 3.3 Screening of *Pseudomonas* specimens for antimicrobial compound (Chloroform Overlay Assay)

S. No	Sample Code	MRSA Zone of Inhibition (mm)			Environmental Isolates	
		1	2	3	Y.B	O.B
1	P-1	30	15	–	25	30
2	P-2	–	–	–	20	–
3	P-3	32	30	–	–	15
4	P-4	36	15	–	20	29
5	P-5	55	15	–	40	45
6	P-6	42	40	30	30	30
7	P-7	44	–	–	40	65
8	P-8	–	–	–	45	44
9	P-9	42	20	–	45	15
10	P-10	49	35	–	–	–
11	P-11	–	38	–	40	42
12	P-12	30	–	–	20	18
13	P-13	–	–	–	–	–
14	P-14	20	25	–	35	–
15	P-15	34	35	25	40	46

16	P-21	–	–	–	–	–
17	P-22	–	–	–	–	–
18	P-23	–	–	–	–	–
19	P-24	–	–	–	–	–
20	P-25299	45	20	–	37	30
21	P-1419	43	–	20	–	–
22	P-24357	40	20	–	35	50
23	P-24244	–	–	–	–	–
24	P-1207	42	–	32	–	14
25	P-1086	40	–	–	–	45
26	P-1843	54	20	20	–	25
27	P-25167	32	25	–	–	18
28	P-298	46	–	–	35	37
29	P-21713	26	–	15	–	37
30	P-23612	35	32	20	18	26
31	P-23811	–	–	–	–	–
32	P-21361	45	–	–	–	46
33	P-22502	56	–	–	–	–
34	P-23498	30	–	30	–	40
35	P-21347	–	–	–	–	–
36	P-1420	23	–	–	–	55
37	P-547	32	–	30	35	32
38	P-23927	–	–	–	–	–
39	P-45	–	–	–	–	–
40	P-46	–	–	–	–	–

Table 3.5 Antibiotics resistance profile of antimicrobial compound produced *Pseudomonas* strains

S. N O	Sample code	Antibiotics resistance profile	
		Resistant	**Sensitive**
1	P-1	IPM, CIP ,TOB, SCF, LEV, CN, AMC, CRO	TZP, FEP, CAZ, PRL, AK, CTX, MEM, PB
2	P-2	IPM, CTX	CAZ, LEV, AK, TOB, FEP, SCF, PRL, TZP, CIP, MEM, PB, CN, AMC, CRO
3	P-3	CIP,IPM, LEV, CTX	TZP, PRL, FEP, TOB, SCF, AK, TZP, CAZ, MEM, PB, CN, AMC, CRO
4	P-4	IPM, CIP, TOB, CAZ, LEV, AMC, CRO	PRL, TZP, AK, SCF, FEP, CTX, MEM, PB, CN
5	P-5	TZP, CIP, IPM, CAZ, PRL, AK, FEP, TOB, TZP, SCF, MEM, CN, AMC, CRO	LEV, CTX, PB
6	P-6	PRL, CIP, CTX	LEV, AK, TOB, CAZ, IPM, FEP, SCF, TZP, MEM, PB, CN, AMC, CRO
7	P-7	LEV, SCF, TOB, CAZ, TZP, AMC	IMP, AK, PRL, CIP, CTX, MEM, PB, CN, CRO
8	P-8	AK, PRL, TOB, CIP, CTX, AMC	LEV, IMP, SCF, TZP, MEM, PB, CN, CRO
9	P-9	TOB, CAZ, FEP, CAZ, LEV, AK, AMC	PRL, IPM, TZP, SCF, CIP, PB, TZP, CTX, MEM, CN, CRO
10	P-10	PRL, AMC	CAZ, TZP, IPM, TOB, CIP, FEP, SCF, AK, LEV, CTX, MEM, PB, CN, CRO
11	P-11	SCF, FEP, CAZ, IPM, CIP, LEV, CTX, MEM, CN, AMC, CRO	TOB, TZP, AK, PRL, PB

12	P-12	-	CAZ, PRL, CIP, SCF, FEP, TOB, TZP, AK, IPM, LEV, CTX, MEM, PB, CN, AMC, CRO
13	P-13	TOB, SCF, PRL, CIP, CTX	IPM, TZP, CAZ, CRO, AMC, AK, LEV, MEM, PB, CN
14	P-14	PRL, AMC	AK, CAZ, SCF, FEP, IPM, CIP5, TZP, TOB, LEV, CTX, MEM, PB, CN, CRO
15	P-15	IPM, AK, FEP, TZP, AK, CIP, TOB, SCF, PRL, CAZ, CTX, AMC, MEM, CN, CRO	LEV, PB
16	P-25299	AMC, PRL, CAZ, CIP	MEM, LEV, TZP, IPM, AK, TOB, CRO, SCF, CTX, PB, CN
17	P-24357	TOB, CAZ, AMC	CN, MEM, SCF, AK, TZP, PRL, LEV, IMP, CIP, CTX, CN
18	P-1419	CRO, AMC, CTX, AK, TOB, SCF, PRL, MEM, CN	TZP, LEV, IMP, CAZ, CIP, PB
19	P-1207	AMC, CTX, PRL, CIP	TZP, PB, AK, SCF, TOB, MEM, CRO, LEV, IMP, CAZ,CN
20	P-1086	MEM, LEV, SCF,VA, CRO, AMC, CTX, AK, TOB, MEM, CN, CRO	PB, TZP, PRL, IMP, CAZ, CIP
21	P-1843	LEV, SCF, VA, TOB, CIP, CTX, AMC, CRO	CN, MEM, SCF, IMP, AK, PRL, CAZ, TZP, PB
22	P-25167	MEM, LEV, SCF, IMP, CIP, CTX, CN, AMC	TOB, MEM, CRO, TZP, AK, PRL, CAZ, PB
23	P-298	AMC, IMP, CAZ, CIP	MEM, TZP, SCF, PB, TOB, CTX, CRO, LEV, AK, PRL, CN
24	P-21713	MEM, PRL, CN,TOB, LEV, TZP, CAZ, IMP, SCF, AMC,	PB, AK, CIP, CTX,

		CRO	
25	P-23612	CTX, AMC, CRO, CN, LEV, PIP, CIP, MEM, CN	IPM, AK, TOB, SCF, PRL, PB, CAZ, TZP
26	P-21361	IMP, TOB, CIP, CTX, AMC, CRO	PRL, CN, TZP, PB, LEV, MEM, AK, CAZ, SCF
27	P-22502	–	FEP, LEV, MEM, TZP, CN, AK, PB, IMP, TOB, SCF, PRL, CAZ, CIP, CTX, CRO
28	P-23498	AMC, CAZ, IMP, TOB, CIP, CTX, MEM	CRO, AK, LEV, TZP, PB, SCF, PRL, CN
29	P-1420	AMC, CN, IMP, TOB, CIP, CTX, MEM	CRO, PB, TZP, AK, LEV, CAZ, SCF, PRL
30	P-547	TOB, AMC	AK, SCF, FEP, ATM, TZP, IPM, CIP, CAZ, PB, CN, LEV, PRL, CTX, MEM, CRO

Antibiotics resistance profiling of Pseudomonas strains

Bar chart showing resistance percentages: CIP5 62%, IMP10 40%, CAZ30 37.5%, PRL100 40%, CN10 37%, TOB10 53%, TZP110 13%.

Legend: Antibiotics resistance pattern for pseudomonas strains

Antibiotics resistance profiling of Pseudomonas strains

Bar chart showing resistance percentages: CTX30 60%, SCF105 33%, MEM10 30%, LEV5 33%, PB300 0%, AK30 13%, FEP30 27%, CRO30 33%.

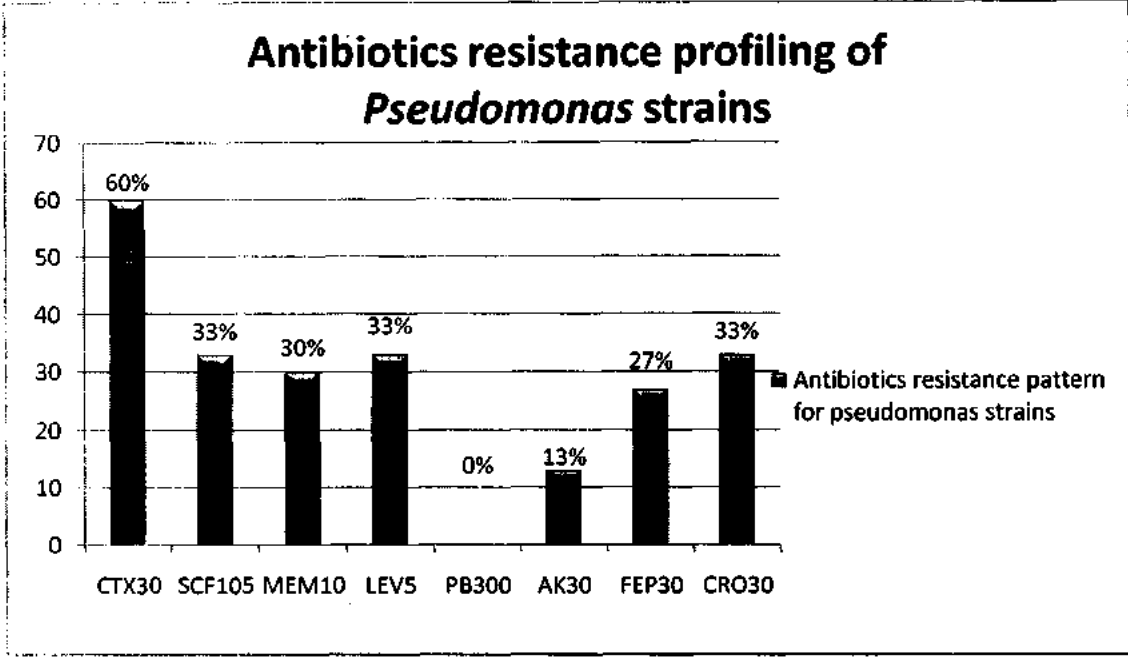Legend: Antibiotics resistance pattern for pseudomonas strains

Figure no 3.1 Resistance percentage of antibiotics discs used against clinical *Pseudomonas* strains

Antibiotics sensitivity profiling of *Pseudomonas* strains

Bar chart with y-axis from 0 to 80. Categories: CIP5 (48%), IMP10 (60%), CAZ30 (63.5%), PRL100 (60%), CN10 (63%), TOB10 (47%), CRO30 (67%). Legend: Antibiotics sensitivity pattern for pseudomonas strains

Antibiotics sensitivity profiling of *Pseudomonas* strains

Bar chart with y-axis from 0 to 120. Categories: CTX30 (40%), SCF105 (67%), MEM10 (70%), LEV5 (63%), PB300 (100%), AK30 (87%), FEP30 (73%), TZP110 (87%). Legend: Antibiotics sensitivity pattern for pseudomonas strains
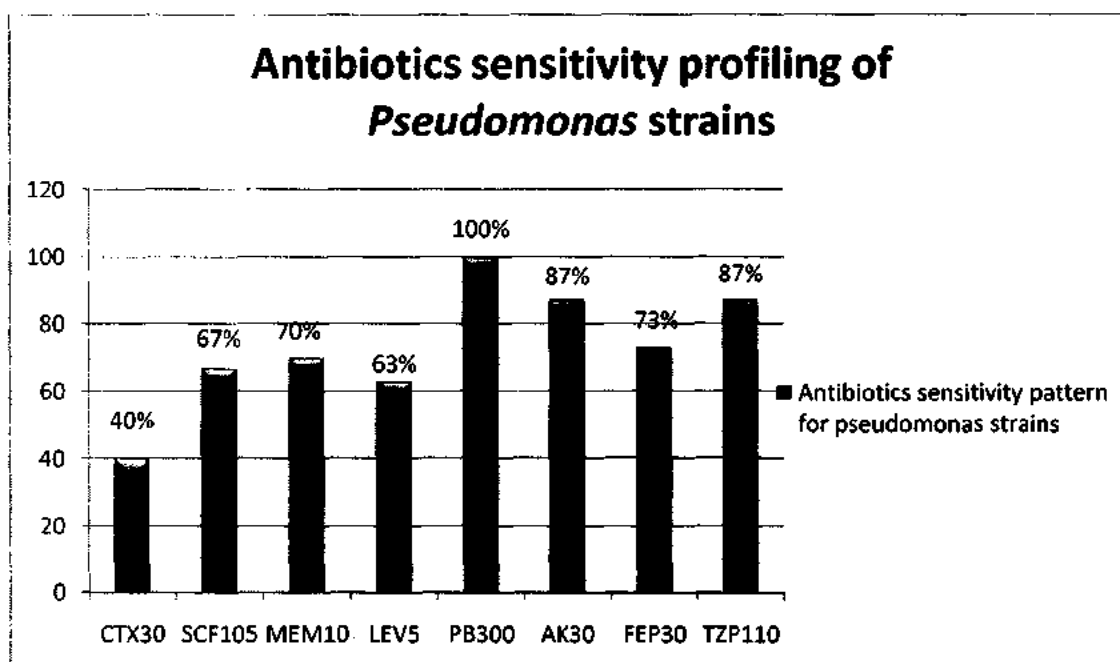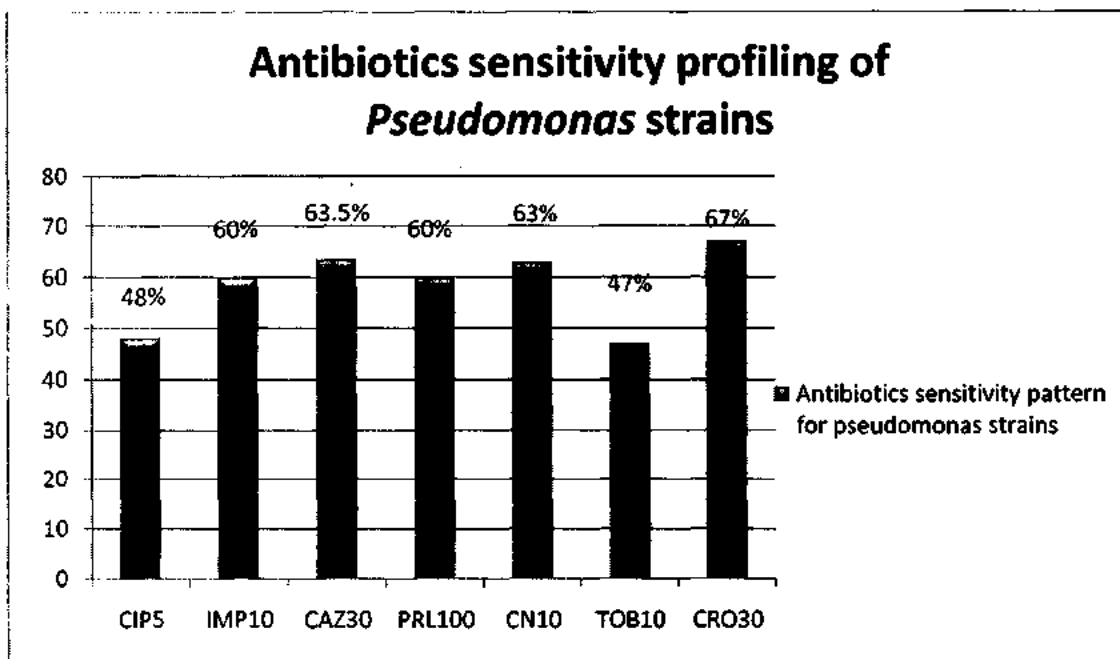
Figure no 3.2 Sensitivity percentage of antibiotics discs used against clinical *Pseudomonas* strains

40

Table 3.6 comparison of zones of inhibition produced by antibiotic and *Pseudomonas* strains against MRSA1, MRSA2, MRSA3

| S. No	Antibiotic	ZONES OF INHIBITION (mm)		
		MRSA1	MRSA2	MRSA3
1	DA2	Intermediate	Resistant	16
2	AZM15	Resistant	Resistant	15
3	STX25	Resistant	Resistant	Resistant
	CIP5	Intermediate	Resistant	Intermediate
4	C30	10	13	13
5	VA30	11	15	12
	TOB10	Resistant	Resistant	Resistant
6	FOX30	Intermediate	Resistant	10mm
7	AMP10	Resistant	Resistant	12mm
8	Pen	Resistant	Resistant	Resistant
9	CLR15	Resistant	Resistant	Resistant
10	TCP	15	Intermediate	10
11	MH30	13	10	11
12	LZP30	15	12	10
13	OXC	10	10	13
14	OFI	9	12	10
S. NO	Pseudo-sample			
1	P-6	42	40	30
2	P-15	34	34	25
3	P-1843	54	20	20
4	P-23612	35	32	20
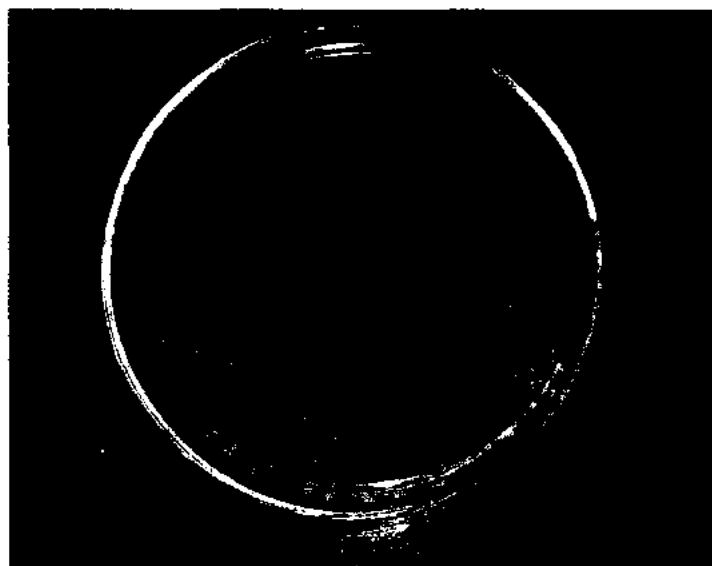
Figure no 3.3 The nutrient agar plate showing antibacterial activity of P-547 against MRSA-1

Figure no 3.4 The trypton agar plate showing antibacterial activity of P-4 against MRSA-1

(Chloroform Overlay Assay)

42

Figure no 3.5 The nutrient agar plate showing antibacterial activity of P-547 against MRSA-1
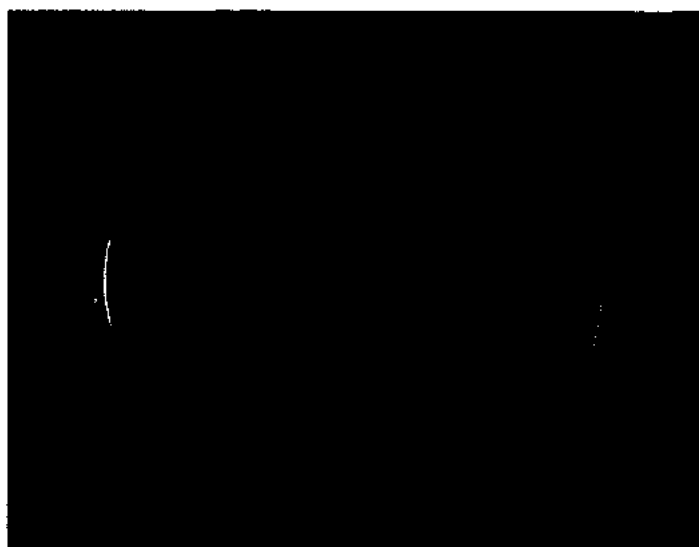(Chloroform Overlay Assay)
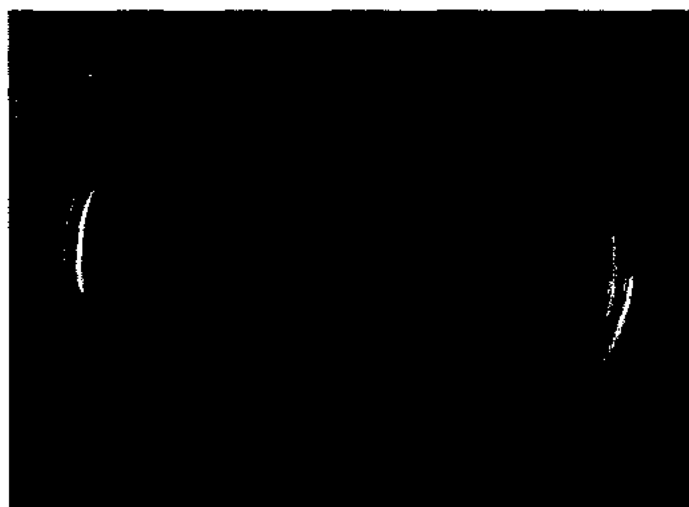
Figure no 3.6 The trypton agar plate showing antibacterial activity of P-11 against MRSA-2

(Chloroform Overlay Assay)

Figure no 3.7 Tthe trypton agar plate showing antibacterial activity of P-15 against MRSA-3

(Chloroform Overlay Assay)
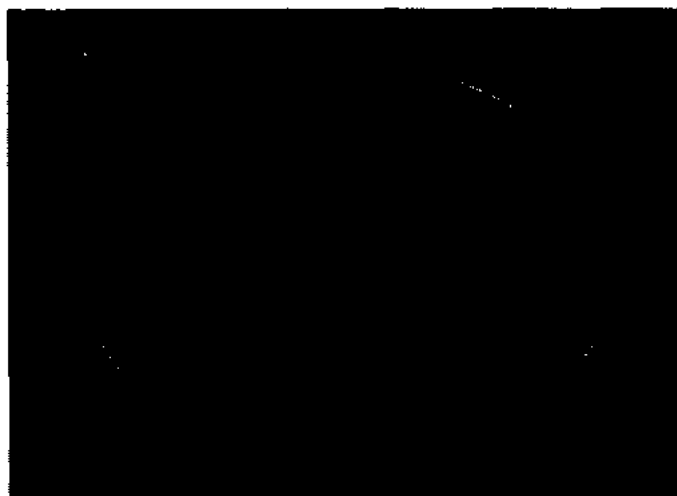
Figure no 3.8 The trypton agar plate showing antibacterial activity of P-5 against yellow
environmental bacteria (Chloroform Overlay Assay)
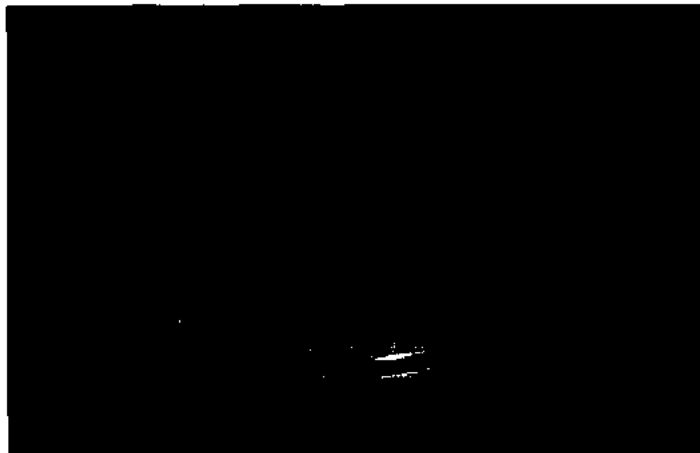
Figure no 3.9 The trypton agar plate showing antibacterial activity of P-1086 against orange environmental bacteria (Chloroform Overlay Assay)

Figure no 3.10 The nutrient agar plate showing dark green, light green and yellowish green pigmented *Pseudomonas* colonies

A

B

C

D
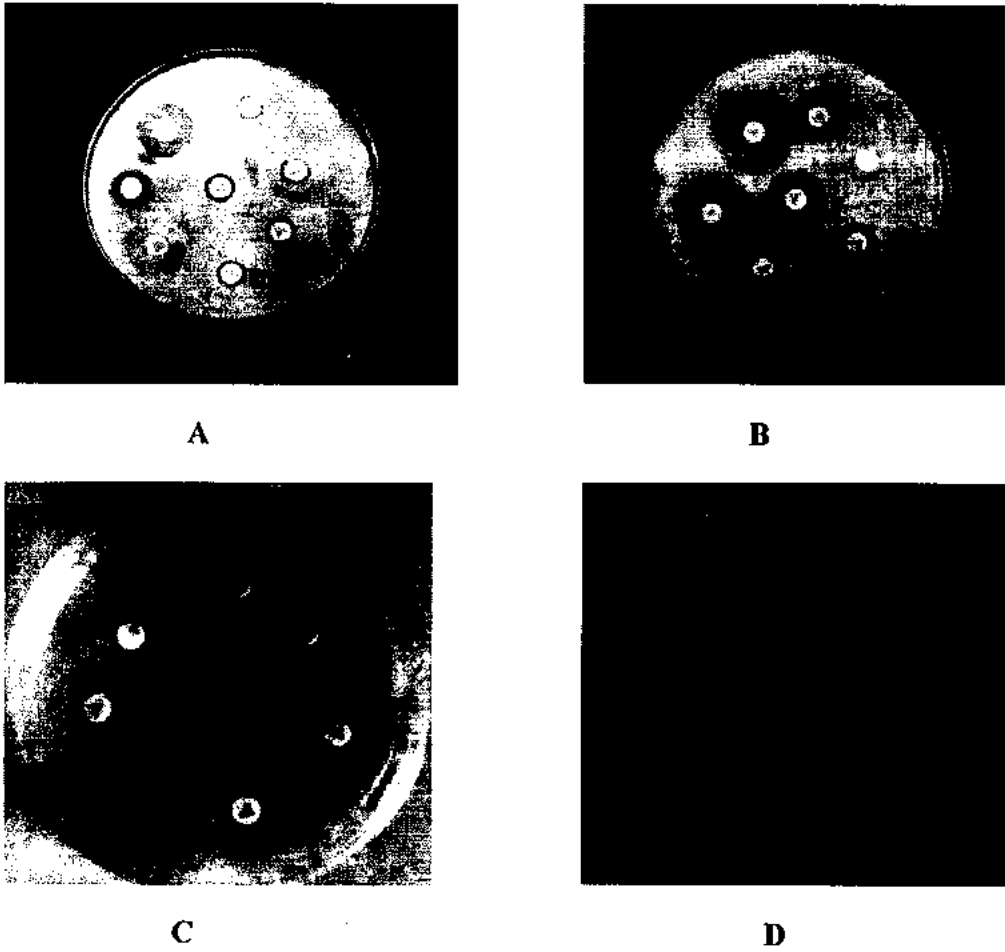
Figure no 3.11 Antibiotics resistance profiles of MRSA-1 (A), MRSA-2 (B), MRSA-3 (C) and *Pseudomonas* strain P-5 (D)
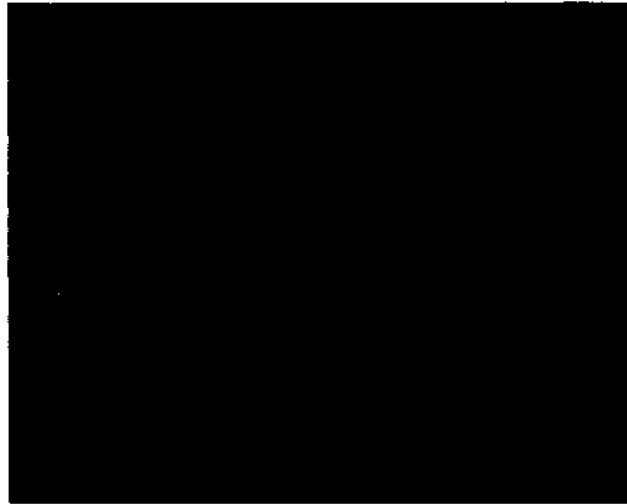
Figure no 3.12 Shows Oxidase Test positive result
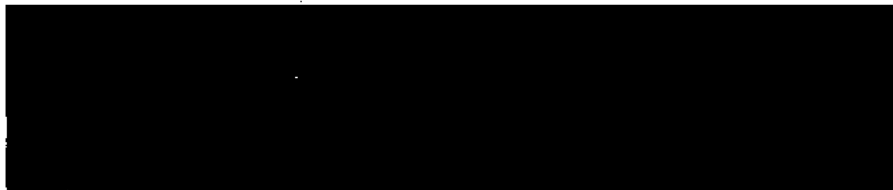
Figure no 3.13 shows Catalse Test. Production of bubbles indicates positive reaction i.e. hydrolyzing of hydrogen peroxide

PCR amplification of 16s RNA gene
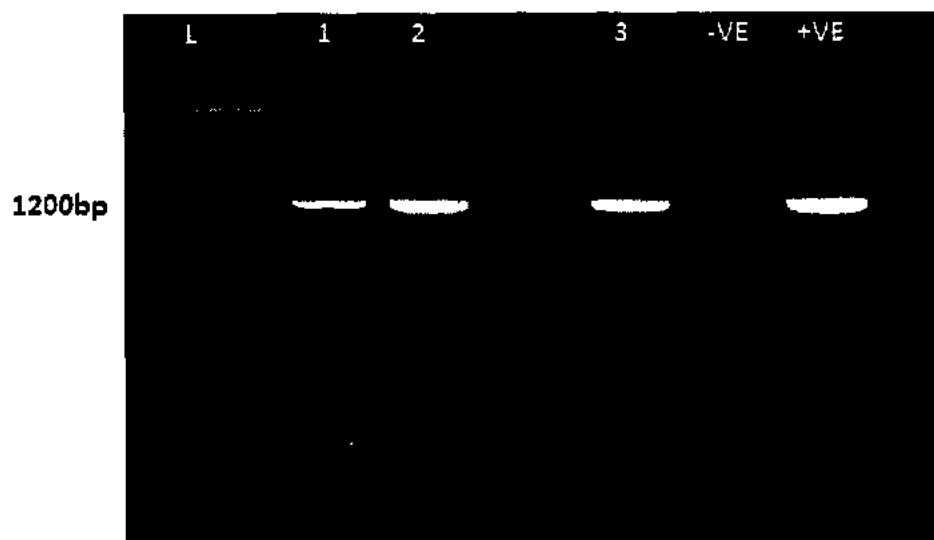
Figure no 3.14 PCR amplification at 1200bp. Lane l: 1kb ladder. Lane: 1, 2, 3 samples positive for *Pseudomonas*, Lane 4: negative control. Lane 5: positive control
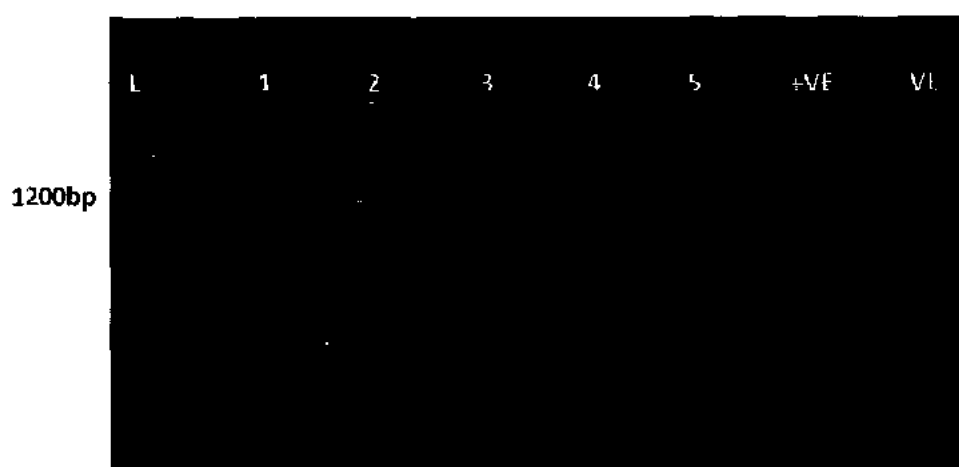
Figure no 3.15 Showing PCR amplification at 1200bp. Lane L: 1kb ladder. Lane 1, 2, 3, 4, 5: samples positive for *Pseudomonas* strain. Lane 6: for positive control. Lane 7: negative control

3.5 Sequencing

Only 6 samples were sequenced by. 16S universal primer was used for partial sequencing (16S rRNA) of isolated *Pseudomonas* strains. Following are the sequences of isolated *Pseudomonas* strains.

TGTTTTGCCCACAAATAAAACTCCAAAGTCGAGCGAAAAAATTCTGCGGGGTCCGG
GACCCGAGGAGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAA
CGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCT
TCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAG
GCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCACGCAGTGGGGAATATTGGACAATG
GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGTAGAAGGTCTTCGGATTGTAAA
GCACTTTAAGTTGGGAGGAGGGCAGTAAGTTATACCTTGCTGTTTTGACGTTACCAC
AGAATAAGCACCGGCTACTTCGTGCAGCAGCCGCGGTAATACGAAGGGTGCAAGCG
TTAATCGGATACTGGGCGTAACCCGAGGGGTCACAATGGTGTAAACCCGGGTAACT
GGAGTGC

Figure no 3.16 partial sequences (521 nucleotide) of strain P-6

GAGATATGAAGGGGGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTA
GGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATAC
GTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGG
TCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAG

Figure no 3.17 partial sequences (241 nucleotide) of strain P-1420

GAATCATGAATCACTCCGTGGTAACCGGGCTTGCGGTTAGTTTAGCTACTTCTGGAG
CAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC
CGGGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAG
ACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCA
ACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATG
ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCC
ACCCGAGGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCA
ACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGA
AGGCACCAATCCATCTCTGGA

Figure no 3.18 partial sequences (441 nucleotide) of strain P-1843

AGACGAAGGCATCTTTGGAGATTGCTGCTGGACTGTTGGGAAAGGAGAGTTTTTACT
AGGATCTGCCTTGTATGCCCCTAACGTCAGGAAACTAACGCTTGGCCCAGGTGGTCC
TGCCGCAGAACGTGTTAGGCCGCCTGACCTCACACTATCAGATGAGCCTAGGTCGG
ATTAGCTAGTTGGCGCGGTAAAGGCCTACCAAGGCGACGATCCGCGACTGGTCTGA
GAGGATGATCAGTCACACTGGAACTGAGACATCGTCCACACTCCTACGGGAGGCAG
CAGTGGACACCTTGGACAATGGGCGAAAGCCTGCTCCAGCCATGCCCCGAAGTCTA
ACCAATCTTCGCACGGGAAGGAATTAAGGTTTCGAGGAAGAACTCTGTTATACATTA
CTCTTGACTTTACCACTGAATAACCCGACTAACTTCACCCAAACCCCCGGTAACTCA
TGGCGCAGCGTGTCTTAATTACTTGG

Figure no 3.19 partial sequences (441 nucleotide) of strain P-9

TAGAGGAGTGATAGATGAGTGGGAGGAGGAACCAGTGGGGGAGCAAGCGGGTGAG

CCCGTATCGGAGTCGGGGGGGTACGGCATAGGTTTTGAAGATTGGGGTATGGGCTA

CGCCGGACGGCCTCTCCGAGGCAGGCTGGGGAGAAAGTGGGGGATCTTCGGACCTC

ACGCTATCCGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCA

AGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACGGGAACTGAGACA

CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC

CTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAG

TTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAA

GCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAAT

CGGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAAC

Figure no 3.20 partial sequences (521 nucleotide) of strain P-8

AACCGGCTGGCTGGAAGGGGTACGGGGGGACGGCCCCCTTGCGGTTAGACTAGCTA

CTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA

CGTATTCACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTC

GAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGC

GGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGG

GCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTT

A

Figure no 3.21 partial sequences (321 nucleotide) of strain P-547

3.6 BLAST Result and Multiple Sequences Alignment

Sequencing of six samples was analyzed. BLAST results showed that P-6 strain isolated from blood had maximum 99% similarity with *Pseudomonas aeruginosa* 16S rRNA gene sequence (EU368174.1). Multiple sequence alignment showed one substitution and three gaps. Similarly BLAST result of strain P-1420 isolated from blood had 100% similarity with *Pseudomonas aeruginosa* strain HE21 (LN624810.1) multiple sequence alignment showed zero substitutions and zero gaps between both strains.

BLAST analysis revealed that strain P-1843 isolated from blood had 98% similarity with *Pseudomonas aeruginosa* strain D3 (JN995664.1). This study showed four substitutions and two gaps differences between strains.

In the same way strain P-9 isolated from urine sample showed 94% similarity with *Pseudomonas aeruginosa* strain LESB65 sequence (CP006983.1). They had a difference of nine substitutions and one gap.

Sequence of strain P-8 isolated from pus sample showed 99% similarity with *Pseudomonas aeruginosa* strain FPVC11 (JQ660564.1). One substitution and zero gaps were found between these two strains.

BLAST analysis revealed that sequence of strain P-547 isolated from pus had 100% similarity with *Pseudomonas aeruginosa* strain NBRAJG70 (EU661692.1). Sequence alignment result did not show any gap and substitution. (Table 3.7)

Table 3.7 Comparison of *Pseudomonas* isolates obtained in this study with previously reported isolates

Isolates	Matched strain	Differences		Percentage of similarity
P-6	EU368174.1	G:1	S: 3	99%
P-8	JQ660564.1	G: 0	S1	99%
P-9	CP006983.1	G: 1	S: 9	94%
P-547	EU661692.1	G: 0	S: 0	100%
P-1420	LN624810.1	G: 0	S: 0	100%
P-1843	JN995664.1	G: 2	S: 4	98%

3.7 Phylogenetic analysis

On the basis of 16S rRNA gene sequence phylogenetic tree of samples P-6, P-8, P-9, P-547, P-1420 and P-1843 and reference strains was constructed by using neighbor joinging method. Reliability of phylogenetic tree was estimated by bootstrapping phylogenetic reconstruction of 1000 replicates as shown in Figure 3.22
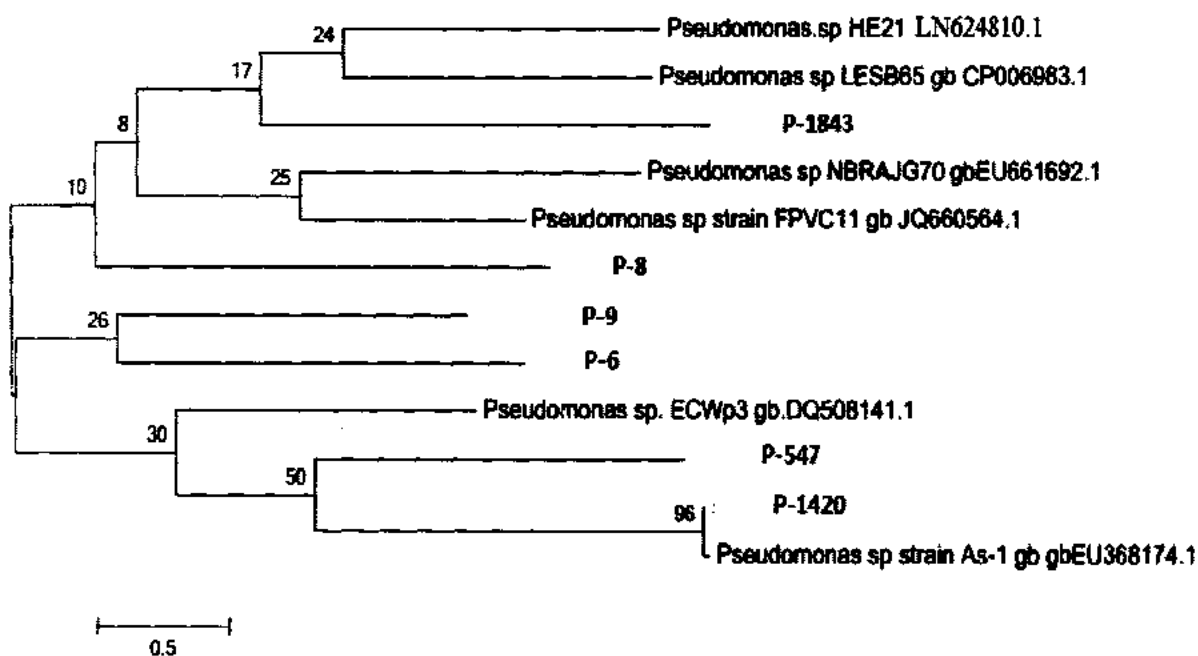

Figure no 3.22 phylogentic tree from published 16S rRNA gene of closely related *Pseudomonas* species (accession numbers) and *Pseudomonas* strains isolated from clinical samples by neighbor joining method of 1000 replicates.

DISCUSSION

4. Discussion

Although our knowledge about *Pseudomonas aeruginosa* species has progressed significantly over the last few years, but in hospitals this bacterium remains a curse, causing fatal and persistent infections dispite of antimicrobial treatment. *Pseudomonas aeruginosa* infections are very hard to treat due to its inherent aptitude to show resistant against much different kind of antibiotics. Different kinds of antibiotics resistance mechanisms exhibit by this bacterium e.g. intrinsic, acquired, and adaptive. Resistance rates are also increasing regardless of combination drug therapies. It has unique habitat, metabolic changes, and complex regulatory controls, it is unlikely that *Pseudomonas aeruginosa* species will ever be entirely eradicate from hospital environment; hence, control preventive methods and early interference remain the most efficient methods for the control of *Pseudomonas aeruginosa* species (Stephenson and Hoch, 2002).

The present study was done during the period of December 2013 to November 2014. In this study, an effort was made to identify the prevalence of antimicrobial compound producing *Pseudomonas* strains and molecularly characterize these strains. In order to conduct this study, 50 samples were screened for the presence of *Pseudomonas* strains and out of 50 samples 40 samples were declared as *Pseudomonas* strains. Morphological characteristics of *Pseudomonas* strains shows that highest ratio of samples were light green pigmented strain eight sample were produced dark green pigments and 10 samples were non pigmented strains. Most number of samples belonged to Gram's negative bacteria almost 86% and 14% Gram's positive bacteria. Different biochemical tests were applied on all the 50 strain and out of 50, 40 samples were catalase positive and while 10 samples shows negative results. TSI test result showed that forty samples were non lactose fermenting, obligate aerobe and can also hydrolyse hydrogen peroxidase. Two samples were lactose fermenting, obligate aerobe and can also hydrolyse hydrogen peroxidase while one sample was lactose fermenting, facultative aerobe and can also hydrolyse hydrogen peroxidase. Catalase, oxidase and TSI test results showed that these tests are reliable and good for the detection of *Pseudomonas* strains.

Pseudomonas strains were screened for the antimicrobial compound producing ability and its activity was checked against methicillin resistant *Staphylococcus aureus* (MRSA) and two environmental isolates. This study extends on previous study of Cardozo *et al.*, 2013 in which the antibacterial activity of a compound obtained from *Pseudomonas aeruginosa* was evaluated

resistance to noncarbapenem ß-lactams. Such resistance is usually related with high production of a naturally producing enzyme cephalosporinase (AmpC).

The present resistance level of commonly used antipseudomonal antibiotics are studied by Bonfiglio *et al.*, 1998 in more than one thousand clinical isolates of *Pseudomonas aeruginosa*. According to their study ceftazidime, amikacin, and ciprofloxacin are, 13.4% and 10.6%, 31.9% are susceptible to *Pseudomonas aeruginosa* respectively.

According to a survey conducted for the SENTRY Antimicrobial Surveillance Programme on Gram-negative bacilli bloodstream infections and rate of incidence and antimicrobial vulnerability level of isolates collected in the United States, Canada, and Latin America also concluded high resistance to third generation cephalosporins and ciprofloxacin (Diekema *et al.*, 1999).

Reynolds *et al.*, 2004 determined antimicrobial vulnerability of the blood pathogens and resistance level of *Pseudomonas aeruginosa* to ciprofloxacin, ceftazidime in the UK and Ireland between 2001-2002. They found out that the resistance rate to ciprofloxacin and ceftazidime was between 4% and 7%.

MRSA1, MRSA2, MRSA3 showed resistance against antibiotics Co-trimoxazol (25µg), Tobramycin (10µg), penicillin and clarithromycin (15µg). antibiotics minocycline (30µg) LZP30, OXC and OFI produce zone of inhibition for MRSA1, MRSA2 and MRSA3. *Pseudomonas aeruginosa* isolated strains also produce greater zone of inhibition against MRSA1, MRSA2 and MRSA3 as compared to the selected antibiotics discs.

Pseudomonas aeruginosa identification has traditionally based on phenotypic methods. This method is still the most precise standard for identification of typical isolates of *Pseudomonas aeruginosa*. In some cases *Pseudomonas aeruginosa* isolates exhibited unusual phenotypic reactions such as in cystic fibrosis (CF) patients. Moreover, biochemical testing is time consuming and also requires extensive handson work by the technologist, both for setup and for ongoing evaluation. Molecular methods have been considered as the most accurate as compared to the phenotypic methods for detection of *Pseudomonas aeruginosa* (Qin *et al.*, 2003).

Among all the genotypic methods, 16S rRNA gene sequencing is one of the most frequently used methods for bacterial recognition (Kim and Jang, 2012). Those bacterial species which are difficult to recognize through common methods can be identified through 16S rRNA gene sequencing in hospitals. However, in case of *Acinetobacter* species, 16S rRNA gene sequencing

is not sufficient to differentiate all the species (Alvarez-Buylla *et al.*, 2012; Janda and Abbott, 2007).

Pseudomonas identification methods through genotyping bypass the problem of different phenotype to provide more correct identification. However, the complex structure and taxonomy uncertain phylogeny, and paucity of genomic sequence data of the many of species within the genus *Pseudomonas* present a hurdle to genotypic identification assays. 16S rRNA the part of the DNA commonly used for taxonomic purposes for bacteria. The identity of all isolates was confirmed by using biochemical Infection testing such as oxidase test, catalase test and TSI agar test (Tripathi *et al.*, 2013).

PCR amplification of 16S rRNA gene was performed and PCR results showed positive results for *Pseudomonas*. Out of all *Pseudomonas* strain that showed positive results, six *pseudomonas* strains were sequenced and the query sequences blasted against the available sequences by using BLAST program on NCBI. Sequencing of isolated strain was analyzed. BLAST results showed that P-6 strain isolated from blood had maximum 99% similarity with *Pseudomonas aeruginosa* 16S rRNA gene sequence (EU368174.1). Multiple sequence alignment showed one substitution and three gaps. Similarly BLAST result of strain P-1420 isolated from blood had 100% similarity with *Pseudomonas aeruginosa* strain HE21 (LN624810.1). BLAST analysis revealed that strain P-1843 isolated from blood had 98% similarity with *Pseudomonas aeruginosa* strain D3 (JN995664.1). Strain P-9 isolated from urine sample showed 94% similarity with *Pseudomonas aeruginosa* strain LESB65 sequence (CP006983.1). Strain P-8 isolated from pus sample showed 99% similarity with *Pseudomonas aeruginosa* strain FPVC11 (JQ660564.1). Strain P-547 isolated from pus had 100% similarity with *Pseudomonas aeruginosa* strain NBRAJG70 (EU661692.1).

In this study phylogenetic affiliation of *Pseudomonas aeruginosa* using 16S rRNA gene sequence analysis was also performed. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Imanishi, 1987). The evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura and Kumar, 2004). The analysis involved 12 nucleotide sequences. Phylogenetic tree was constructed on the basis of 16S rRNA gene sequence of P-6, P-8, P-9, P-547, P-1420 and P-1843 and reference strain by using neighbor joinging method. Reliability of phylogenetic tree was estimated by bootstrapping phylogenetic reconstruction of 1000 replicates.

According to tree, *Pseudomonas aeruginosa* strain seq strain 1420 and *Pseudomonas aeruginosa* reference strain AS-1 with accession number gb EU368174.1 are in one cluster with a bootstrap value of 1000 showing 96% reliability of this cluster.

Conclusion

In our study, *pseudomonas* strains isolated from clinical samples were declared as *Pseudomonas aeruginosa* after sequencing. It is also concluded that *Pseudomonas aeruginosa* became resistant to a broad range of antibiotics. Antimicrobial resistance levels are increasing day by day therefore great care is required while prescribing antibiotics and appropriate use of antibiotics. Continuous observation is must on changes in rates of resistance among *Pseudomonas aeruginosa* infections. A vigilant monitoring of antibiotics usage in hospitals is essential to recognize situations in which prescription patterns are helpful in the progress of antimicrobial resistance. There should be a continuous monitoring at national, regional level as these inspections are crucial to provide clinicians with information for prescribing correct treatment regimens.

The results of our findings suggest that *Pseudomonas aeruginosa* produce secondary metabolite which could have inhibitory effect against methicillin-resistant *staphylococcus aureus* (MRSA) strains that are responsible for infectious diseases in humans and other animals. This compound can be used as a good substitute in the treatment and control of MRSA infections.

Future Prospectus

In future, antimicrobial compound produced by *Pseudomonas aeruginosa* against Methicillin-resistant *Staphylococcus aureus* (MRSA) will be isolated. Its chemical structure will be determined. Genes of *Pseudomonas aeruginosa* responsible for the production of antimicrobial compound against environmental isolates and Methicillin-resistant *Staphylococcus aureus* (MRSA) will be find out.

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APPENDICES

APPENDICES

Media for Bacterial Growth

Appendix I. Composition of Nutrient agar medium

Ingredients	Quantity
Lab-lemco powder	1.0g
Yeast extract	2g
Agar	15g
Peptone	5g
Sodium chloride	5g
Distilled water	1000ml
pH :	7-7.5

Appendix II. Composition of Trypton agar

Ingredients	Quantity
Trypton	10g
Yeast extract	5g
Agar	30g
Sodium chloride	10g
Distilled water	1000ml
pH	7.5

Appendix III. Composition of Trypton broth

Ingredients	Quantity
Trypton	10g
Yeast extract	5g
Sodium chloride	10g
Distilled water	1000ml
pH	7.5

Appendix IV. Recipe of 10X and 1X phosphate Buffer saline

10X phosphate buffer saline (PBS) [pH. '		
S. No	Ingredients	g/1000ml
1	NaCl	87.6625g
2	Na_2HPO_4	11.9375g
3	KH_2PO_4	2.5625g
4	Deionized water	1000ml
1X phosphate buffer saline (PBS)		
S. No	Ingredients	500ml
1	10XPBS	50ml
2	Deionized water	450ml

Appendix V. Reagents for sequencing reaction

Sequencing reaction mixtures		
S. No	Ingredients	Volume
1	Purified PCR product	5µl
2	Big dye	4µl
3	Primer (forward and reverse)	1µl
4	Total	15µl

Appendix VI. Conditions for sequencing reaction

Sequencing reaction conditions				
S. No	Steps	Temperature	Time	No. of cycles
1	Initial denaturation	96°C	1min	25X
2	Denaturation	96°C	15sec	
3	Annealing	94°C	4min	

Appendix VII. PCR (polymerase chain reaction)

Reaction mixture			
S. No	Ingredients	Stock concentration	Volume (µl)
1	10XPCR buffer	10X	2µl
2	MgCl$_2$	50mM	0.6µl
3	dNTPs	10mM	1.6µl
4	Taq DNA polymerase	5U/µl	0.4µl
5	Primers (each)	5µM	0.6µl
6	DNA		1µl
7	ddH$_2$O		13.2µl
8	Total		20µl

Appendix VIII. PCR conditions

S. no	steps	Temperature (C°)	Time	Cycles
1	Initial denaturation	94°C	4min	1X
2	denaturation	94°C	45sec	35X
3	Annealing	59°C	45sec	
4	Extension	72°C	45sec	
5	Final extension	72°C	10min	1X

Appendix IX. list of primers used 16S rRNA gene amplification

Primers used for 16S rRNA gene amplification		
S. no	Primer	Sequence
1	16S rRNA- F	GAGTTTGATCCTGGCTCAG
2	16S rRNA-R	AGAAAGGAGGTATCCAGCC

Appendix X. Solutions and Buffers for DNA Extraction

TE Buffer (10mM Tris, 1mM EDTA, pH-8		
S. no	Ingredients	gm/ 100ml
1	Trizma base	0.121
2	EDTA	0.037
3	Distilled water	100ml
5X TBE		
S. No	ingredients	gm/ L
1	Tris base	54.00
2	Boric acid	27.50
3	Sodium EDTA	4.63
4	Ditilled water	1000ml
1X TBE		
S. no	Ingredients	1000mL
1	5X TBE	200ml
2	Distilled water	800ml

Appendix XI. Names of Antibiotics Used To Find Out Resistance Pattern

S.NO	Disc	ANTIBIOTIC
1	LEV5	Levofloxacin 5
2	IMP10	Imipenem 10
3	AK30	Amikacin 30
4	SCF105	sulbactam-cefoperazone
5	PRL100	Piperacillin 100
6	CAZ30	Ceftazidime
7	TZP110	Tazobactam
8	CIP5	Ciprofloxacin
9	FEP30	Cefepime
10	TOB10	Tobramycin
11	CTX30	Cefotaxime
12	MEM10	Meropenem
13	AmC30	Amoxicillin
14	CRO30	Ceftriaxone
15	CN10	Gentamicin
16	PB300	Polymixin B

Appendix XII. DNA concentration. Nanodrop values and dilution

sample id	N.A.CONC ug/ml	260/280	40ng/ul*100/dna conc ug/ml	d.water
P-1	1157.5	1.92	3.455723542	96.54427646
P-2	21.7	1.74		
P-3	29.6	1.55		
P-4	63.3	1.12	63.19115324	36.80884676
P-5	75.9	1.71	52.70092227	47.29907773
P-6	45.2	1.16	88.49557522	11.50442478
P-7	49.9	1.31	80.16032064	19.83967936
P-8	1068.2	1.67	3.744617113	96.25538289
P-9	182.5	1.92	21.91780822	78.08219178
P-10	13.9	2.02		
P-11	347.2	1.77	11.52073733	88.47926267
P-12	223	1.98	17.93721973	82.06278027
P-13	432.1	1.87	9.257116408	90.74288359
P-14	917.2	1.85	4.361098997	95.638901
P-15	254.1	1.92	15.74183392	84.25816608
P-21713	1525.9	2.09	2.621403762	97.37859624
P-298	818.9	2.04	4.884601294	95.11539871
P-23612	48.9	1.91	81.799591	18.200409
P-1207	1431.9	2.09	2.793491166	97.20650883
P-1419	1106.8	2.06	3.614022407	96.38597759
P-25357	1269.5	2.03	3.15084679	96.84915321
P-21361	941	2.03	4.250797024	95.74920298
P-1843	713.9	1.95	5.603025634	94.39697437
P-25299	1299.2	2.02	3.078817734	96.92118227
P-23498	446.2	2.05	8.96458987	91.03541013
P-547	1067.6	2.07	3.746721619	96.25327838
P-22502	842.3	1.83	4.748901816	95.25109818
P-1420	682	2.07	5.865102639	94.13489736
P-1086	905	2.07	4.419889503	95.5801105
P-25167	1164.7	2	3.43436078	96.56563922