IN THE NAME OF ALLAH, THE BENEFICENT
THE MERCIFUL

Read! And thy Lord is Most Honorable and Most Benevolent,
Who taught (to write) by pen, He taught man that which he knew not

(Surah Al-Alaq 30: 3-5)
Al-Quran

CHARACTERIZATION OF ANTIBIOTIC PRODUCING SOIL MICROORGANISMS AGAINST HUMAN PATHOGENIC BACTERIA, ISOLATED FROM HOSPITILIZED PATIENTS IN KHYBER PAKHTUNKHWA

Riaz Muhammad

A thesis submitted to the University of Peshawar in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology and Microbiology

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CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY
UNIVERSITY OF PESHAWAR

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This dissertation is submitted by RIAZ MUHAMMAD as partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology and Microbiology

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This thesis titled *"Characterization of Antibiotic Producing Soil Microorganisms against Human Pathogenic Bacteria, Isolated From Hospitalized Patients in Khyber Pakhtunkhwa*" submitted by Riaz Muhammad is hereby approved and recommended as partial fulfillment for the award of Degree of Doctor of Philosophy in Biotechnology and Microbiology

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Dedication

I dedicate this humble effort of mine to Hospitalized patients

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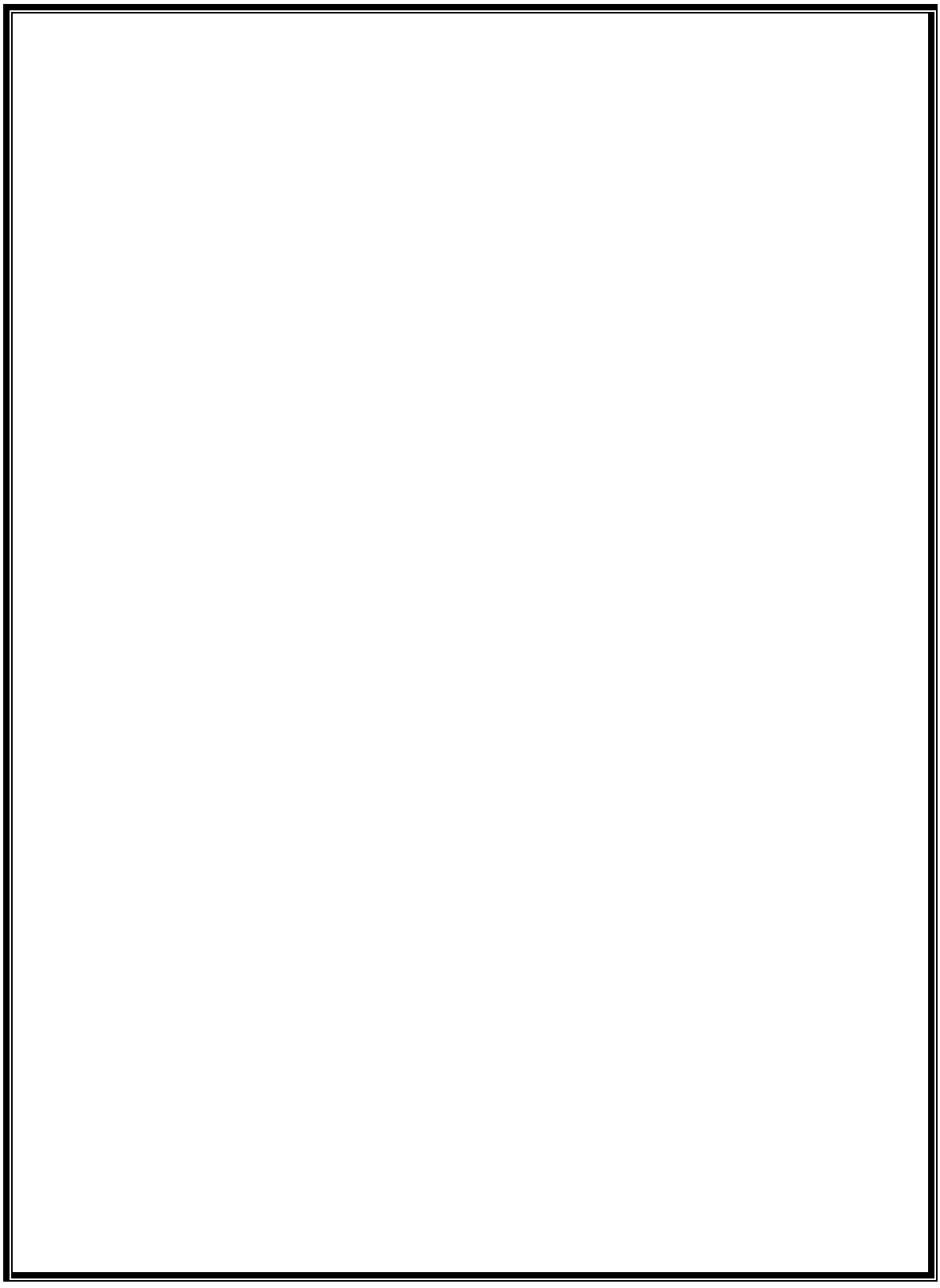

Summary

Pathogenic bacteria are medically very important as they caused various diseases, develop resistance against antibiotics and that is why require great concern worldwide. Most prominent antibiotic resistant pathogens are Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin intermediate *Staphylococcus aureus* (VISA), Vancomycin resistant *Staphylococcus aureus* (VRSA), multi drugs resistant *Pseudomonas aeroginosa*, extended spectrum beta-lactamases producing *Escherichia coli* and *Enterobacteraciae spp.* which cause hospital acquired and community acquired infections. Methicillin and Oxacillin were considered as potent antibiotics against *S. aureus* infection but unfortunately more than 50% *S. aureus* resistance to these drugs have been reported from different countries recently. Now Vancomycin is the drug of choice for the treatment of MRSA infection but unfortunately due to the misuse of antibiotics VRSA have been recently evolved.

In Khyber Pakhtunkhwa pathogenic bacteria have developed resistance to mostly prescribed antibiotics. According to our survey mostly Oxacillin, Meropenem, Amikacin, Ciprofloaxacin, Ofloaxacin, Piperacillin/Tazobactam and Vancomycin antibiotics are prescribed against bacterial infections in hospitalized patients. Bacterial resistance to these antibiotics has also been reported from different geographical locations of Pakistan. Drug resistance is a serious problem which is often a consequence of the failure of control strategies. In the current scenario our options to deal with the antibiotic resistant bacterial pathogens limited or in-effective. As the resistance evolved comparatively rapidly due to the irrational antibiotic use, therefore we expect a much faster evolutionary rate in a third world country like Pakistan. The problem of resistance could be tackled in several ways including synthetic modification in the existing drugs or looking for novel compounds for neutralizing the threat.

In this study human blood, urine, High Veginal Swab (HVS), Cerebrospinal Fluid (CSF), sputum and pus samples were collected from hospitalized patients admitted at different wards of Lady Rading Hospital (LRH) Peshawar. Pathogenic bacteria were isolated using different selective and differential media, after isolation of pathogens, were identified morphologically and biochemically according to standard protocol. Antibiotic sensitivity

assay were carried out for the isolated pathogenic bacteria through Kirby-Bauer method. Furthermore test for extended spectrum beta lactamase detection was also performed through double disc synergy method.

Soil samples were collected from different localities of Khyber Pakhtunkhwa (KPK) for isolation of antibiotic producing microorganisms against isolated pathogenic bacteria through serial dilution technique. Selected microorganisms were identified morphologically and biochemically according to standard protocols. One most potent antibiotic producing microorganisms was selected for optimization of different parameters for maximum production of antibiotics. Extraction of antibiotic was also carried out using different solvent system. At last minimum inhibitory concentration was determined for extracted secondary metabolites against multi drugs resistant pathogenic bacteria.

Total (542) pathogenic bacteria were isolated from (1296) hospitalized patients' samples. The prevalence rate of pathogenic bacteria were *Escherichia coli* (43.91%) followed by *Staphylococcus aureus* (29.88%), *Pseudomonas aeroginosa* (15.68%), *Salmonella. typhi* (2.95%), *Enterococcus spp.* (2.21%), *Proteus mirabilus* (1.29%), *Proteus vulgarus* (1.10%), *Streptococcus pneumoniae* (1.10%), *Klebessiella spp.* (1.47%) and *Enterobacter spp.* (0.36%). Majority of *E. coli* were isolated from urine (106) followed by pus (76) HVS (40), blood (10) and cerebrospinal fluid (5). A total of 238 strains of *E. coli* were isolated. The infection from *E. coli* in female (52.93%) was higher than male patients (47.03%). The resistance pattern of various antibiotics against *E. coli* were Imipenem (2.94%), Cefoperazone/Sulbactam (7.5%), Piperacillin/Tazobactam (8.4 %), Meropenem (3.7%) and Amikacin (22.6%) while less potent antibiotics were Cephradine (96.63%), Cefuroxime (85.29%), Cephtriaxone (78.15%), Ceftazidime (75.21%), Coamoxiclave (72.68%), Cefotaxime (70.58%), nalidixic acid (68.48%). Total fifteen Extended Spectrum Beta lactamases (ESBL) producing *E. coli* were characterized the resistance pattern revealed Cephradin (100%), Gentamicin (100%), Coamoxiclave (100%), Pipemedic acid (100%), Nalidixic acid (100%), Cefurixine (100%), Cefotaxime (93.33%), Meropenem (0%) and Imipenem (0%).

A total of 161 *S. aureus* strains were isolated from samples of hospitalized patients. *S. aureus* Isolated from pus (141), HVS (15) and blood (5) samples out of the 161 isolates. Female patients (50.31%) were more infected from *S. aureus* than male (49.68%). Total (86) Methicillin Resistant *S. aureus* (MRSA) strains (53.41%) were found from all 161 isolates. Antibiotic resistance pattern showed that potent antibiotics were Vancomycin (0%), Taecoplanin (0%), Linezolid (1.20%), Fusidic acid (8.60%) while higher resistance was noted against Cotrimixazol (94.19%), Klathromycin (71.42%), Levofloaxacin (66.45%), Sparfloaxacin (65.56%) and Ciprofloaxacin (65.21%).

In this study (85) *P. aeroginosa* were isolated from Pus (80%), Urine (6%), blood (6%), ear swab (6%) and HVS (2%) specimens. Out of (85) isolated *P. aeroginosa* isolates from male patients were (67.05%) and female hospitalized patients were (32.94%). Most potent antibiotic resistance pattern revealed that Meropenem (7.05%) Imipenem (8.22%), Piperacillin/Tazobactam (10.58 %), and Cefoperazone/Sulbactam (17.64%).

For alternative control strategies of bacterial resistance, *Aspergillus ochraceus RM 82* recovered from soil samples was identified morphologically had potent activities against Multi Drugs Resistant (MDR) human pathogenic bacteria in the primary screening.

Total (220) actinomycetes were isolated from soil samples. All isolated actinomycetes were identified morphologically, microscopy of actinomycetes revealed filamentous rod shaped and spore formation of various arrangements after staining procedures. Total (8) most potent actinomycetes were identified, biochemically and through using different International *Streptomyces* Project (ISP) media, these all identification tests revealed that tested actinomycetes belongs to Genera *Streptomyces*.

In the primary screening for antibiotic producing abilities 35 isolated actinomycetes were found active against MDR pathogenic Gram positive and Gram negative isolated bacteria from hospitalized patients. Furthermore 35 actinomycetes were selected for secondary screening, 27 actinomycetes were found to be secondary metabolites producers against multi drugs resistant pathogenic bacteria and ATCC bacterial strains. Fourteen actinomycetes namely RMN1 to RMN14 had significant bioactive potentials. One isolates of actinomycete RMN5 producing exclusively narrow spectrum bioactive

secondary metabolites against *E. coli* at the range of 13 mm zone of inhibition. Actinomycetes RMN6 producing broad spectrum bioactive metabolites tested against ATCC bacterial culture and multi antibiotic resistant bacteria. Which had anti-*E. coli* ATCC 25922, anti-*P. aeroginosa* ATCC 27553, and anti- *S. aureus* ATCC 25923 activities at the range of 14, 13 and 19 mm zone of inhibition respectively. While against isolated bacterial pathogens *E. coli*, *P. aeroginosa* and *S. aureus* (MRSA) 15, 18 and 23 mm zone of inhibition was recorded respectively.

Most potent isolate of *Streptomyces* RMN6 was selected for further studies. Different conditions were optimized for RMN6 in batch fermentation in which pH 8, 144 hours incubation period, 29 ^{0}C, 7.5% inoculum size, 3% D-glucose concentration and 160 rpm speed of shaking orbital incubator were suitable for maximum activity against selected tested bacterial pathogens. Maximum activity was recorded after conditions were optimized against *Micrococcus luteus* ATCC 10240 and *Staphylococcus aureus* (MRSA clinical isolate) was 32 mm and 28 mm zone of inhibition respectively.

Four spots were developed on Thin Layer Chromatography (TLC) plates observed under UV light at 254 nm and also observed along with iodine vapors. Our results showed antimicrobial activities by two tested spotted compounds which indicate synergistic activities of compounds against tested bacteria. The minimum inhibitory concentration values were in the range of 110 to 135 µg/ml against *Staphylococcus aureus* (MRSA), *Escherichia coli* ESBL (producer), *Pseudomonas aeroginosa* (MDR) and *Salmonella typhi* (MDR).

Our results revealed that antibiotic resistant bacteria are evolving against most potent commercially available antibiotics so we need emergency measures to avoid the misuse of antibiotics. On another hand our results revealed that the soil of KPK exhibit potent microorganisms which produced novel types of secondary metabolites active against MDR pathogenic bacteria. These bioactive compounds can be further purified and can be used as a lead drug for best alternative to replace the existing antibiotics for control of resistant pathogenic bacteria in future.

Our results revealed that bacterial resistance against the available antibiotics is increasing over times. Drug resistance species are of great concern worldwide as they cause high morbidity and mortality and hence more economic burden. These resistant species are not

only a threat for the developing countries, but also equally fatal for developed world. Because of globalization, various means of communication have made it convenient for the pathogens to travel across borders.

Introduction

In broader definition, antibiotics are chemically heterogeneous group of small organic molecules from microbial origin which at diluted form are harmful to the metabolic processes and growth of other microorganisms [1]. Drugs that have been prepared by chemical methodologies in the laboratory are called synthetic drugs while those which extracted from bacteria and fungi referred as antibiotics [2]. Antibiotics can be extracted from microorganisms fermented broth culture and can be modified enzymatically or chemically in the laboratory [3-4]. Antimicrobial drugs can be categorized on the basis of targeted specificity. Broadly narrow spectrum antibiotics are effective against a specific group or type of bacteria, such as potent only against Gram negative or Gram positive pathogenic bacteria. On the other hand, broad spectrum chemotherapeutic agents are effective against wide range of pathogenic microorganisms, Gram negative, Gram positive bacteria and fungi. The potency of recommended antibiotics differs with the site of infection in the body, and the absorption ability of antibiotics to reach that location of infection, more specifically the ability of causative bacteria to resist the used antibiotic. The antibiotic which killed bacteria called bactericidal while those antibiotic which inhibit the growth of bacteria called bacteriostatic. Oral intake of antibiotic is the best approach when potent, while antibiotic through intravenous injections are recommended for more serious conditions of infections. Antibiotics can be used some times topically in the cases of eye or skin bacterial infections in the form of eye drops or ointment. On the basis of antibiotic action against specific microorganisms can also sometimes categorized as *anti-Pseudomonas aeroginosa*, *anti-Escherichia coli*, which mostly depends on the

sensitivity of tested organisms and most commonly caused infections in human population [5].

Industrially antibiotics are produced through the process of fermentation. In industrial fermentation process antibiotic producing microorganism is grown in large container containing 100,000 to 150,000 broth media. All essential parameters required for maximum production of antibiotic are monitored very wisely and kept at optimum ranges, in case any parameter changed from normal optimal value then that parameter adjusted at the same time. The proper population size of producing microbes necessarily is in controlled ranges to produce maximum yield of secondary metabolites in the form of antibiotics before the microbial cell die (Fig 1). When the process of fermentation completed then the antibiotics are extracted and purified to solid crystalline form. It can easily be carried out for those antibiotic products which are soluble in organic solvent adjusted system. If not then, it must be separated first by chemical precipitation, adsorption or through ion exchange methodologies. Microorganisms choose for industrial production of antibiotics is rarely wild type due to less production ability. To obtain the maximum production of antibiotics industrially, important microorganisms are genetically modified to enhance the production ability. Ultra violet radiations, x rays and chemical mutagens are usually used to mutate the wild type antibiotic producing microorganisms. Due to mutation and selection of higher yielding strains over many generations, can increased yield more than 20 times. Another methodology used for higher yield of antibiotics is amplification of gene. Through this method copy of genes coding for proteins involved in antibiotic production can be inserted back in to a cell

through vector (plasmid). The process is effective, if retest produced antibiotic for potential human and animal toxicity and their potency [6].

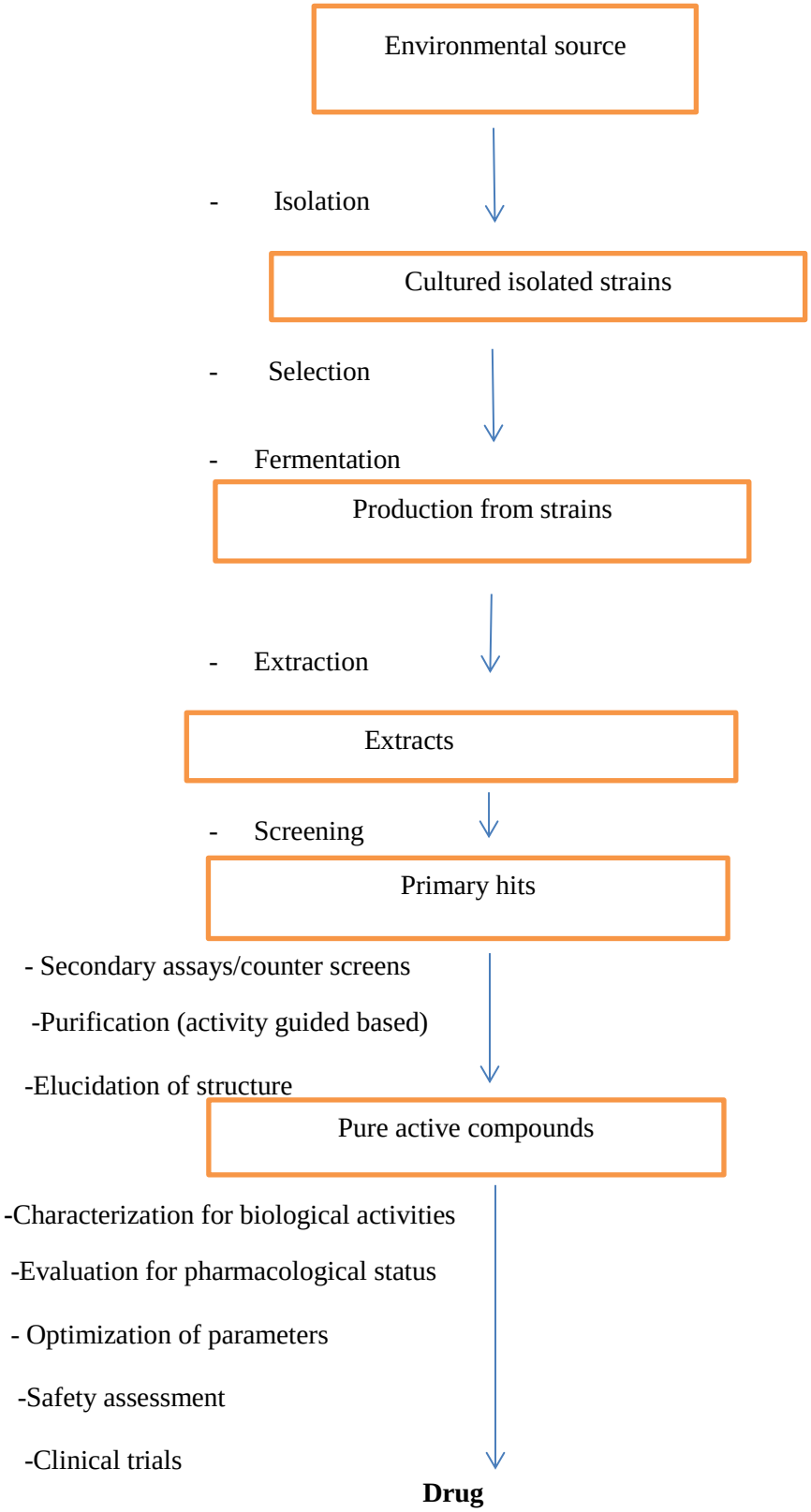

Fig 1. The process of antibiotic from microbial natural product [7]

1.1 Antibiotic resistance

The bright picture of early successful discoveries of antibiotics became blurred due to the threats evolved from the resistant pathogenic bacterial strains. Resistance to drugs presents global public health concerns that comprise all major antimicrobial agents and clinically important pathogens. Now medically important pathogenic bacteria have developed resistance against them not only to single therapeutic agents but also towards many drugs due to the misuse of antibiotics in the last several decades [8].

Antibiotic resistance continuously occurs in human pathogenic bacteria naturally, but the phenomena of resistance in pathogenic bacteria caused huge burden on human population. Due to antibiotic resistance the available antibiotics for the treatment of infectious diseases render ineffective [9]. When microorganisms show resistance to more than one drug these phenomena is referred as multi drugs resistance (MDR). The treatment of multi drugs resistant pathogenic bacteria are very difficult due to inappropriate selection of antibiotics. Proper informatory knowledge of pattern of resistance at regional level and international level; helped physician in an appropriate selection of antimicrobial agents to control MDR pathogens [10]. Antibacterial resistance to antibiotics is either genetically intrinsic or acquired. Intrinsic resistance reflects the activity range of antimicrobials as it is the inherent ability of microorganisms and always persists. Bacterial resistance may occur through mutation in the genetic materials or can be gained from other resistant bacteria through reception of genetic materials [11]. This is due to the misuse of antibiotics in hospitals, self-medication by patients and in agriculture sectors. Bacterial pathogens can easily be transferred from one place to another place via air current, water and other inanimate objects, even from one to another individual.

Bacterial pathogens are present everywhere in the environment and we can easily become a victim of them because the entry of these pathogens is possible through ingestion along with food and inhalation during respiration [9]. Several strategies were developed to decrease or to reverse resistance in pathogens by reducing antibiotic prescription and selected antibiotic prescription in children. By applying these strategies the rate of resistance pattern has been reduced up to 23% in the study period of 1995 to 2000 in the age groups less than three years and above. Public awareness started in UK regarding the use of antibiotics, to stress on the decreased prescriptions. Large numbers of developed countries including UK imposed regulation on the utilization of antibiotics in veterinary [12]. Mutation in bacterial genes which causes resistance usually becomes the loss of fitness and slows down the growth of pathogenic microorganisms. So, it was considered that removal of antibiotics would become the selection against these resistant bacteria during competition with non-resistant bacteria. These strategies unluckily were non workable because bacteria sometimes acquired another type of mutation called compensatory mutations that caused rapid growth than sensitive bacteria. The selective advantage of sensitive bacteria and the selection against resistant mutation was eliminated. To control the resistant pathogens, researchers and pharmaceutical companies must permanently evaluate resistance pattern and test new antimicrobial compounds; in order to provide effective drugs in the market. Unfortunately, pharmaceutical companies focused on finding antibiotic groups similar to the antimicrobials available in the market. Due to the use of similar groups of antibiotics, microorganisms can easily develop resistance by adopting similar mechanisms of action, to which they have already resistant [12].

Pathogenic bacterial strains became resistant through two primary ways.

1- By mutation

2- Through horizontal gene transfer in which resistant genes transfer from one microorganism to another by conjugation, transduction and transformation. Bacteria can take DNA to become antibiotic resistant by gaining resistant genes from other antibiotic resistant bacteria. This mechanism of exchanging DNA, containing resistant genes is required for the survival of bacteria in extreme environment like hospitals [13].

Multi drugs resistant bacteria are largely found in hospitals but also largely spread in the community settings. Some of these pathogens became resistant to large numbers of antibiotics. Infections caused by such type of pathogenic bacteria causes complication in hospitalized patients due to the unavailability of suitable potent drugs to treat patients properly [14].

To treat old diseases we need new antibiotics because of emerging resistant bacteria, but also to treat new diseases new antibiotics are needed. In the last two decades new diseases like legionnairs disease, lyme disease and toxic shock syndrome (TSS) were emerged. We are only become able to know the pattern of susceptibility and resistance to available antibiotics among new pathogens causing new diseases. Broad pattern of resistance were evaluated among these pathogens to different available antibiotics. So, we will soon need new antibiotics to replace the available one that are effective now against such type of bacteria. In the selective environment like hospitals, resistance begins to emerge among them [15].

1.2 Antibiotic resistance to *Staphylococcus aureus*

Staphylococcus aureus became resistant to many antibiotics. Due to the utilization of potent antibiotic indiscriminately *S. aureus* became resistant to them. For example, penicillin was effective against *staphylococcal* infection but the bacteria became resistant due to the misuse of this antibiotic. *S. aureus* produce penicillinase enzyme which break down the beta lactam ring of penicillin and make it ineffective. For the first time penicillin resistant *S. aureus* bacteria was reported in 1945 revealing the production of beta lactamase enzyme production [16]. Penicillin resistant *S. aureus* bacterial infections were treated by Methicillin, Oxacillin and Cloaxacillin antibiotics, which were developed soon after penicillin resistant bacteria. Methicillin was introduced as a drug of choice in 1959, but unfortunately two years later Methicillin resistant *S. aureus* (MRSA) strains were reported in England [17]. MRSA developed resistance to Penicillin, Methicillin and other specific narrow spectrum antibiotics [17]**.** MRSA was reported from United Kingdom in 1961, is now wide spread throughout the world in community settings and more specifically in the hospitalized patients. MRSA is also called Oxacillin Resistant S. aureus (ORSA) because of resistant to Oxacillin antibiotic. In spite, MRSA reported earlier no proper attention was given to it even in hospital settings up to 1990, when an explosion of MRSA prevalence was reported from different parts of the world in hospital settings [18]. In the recent years highest prevalence of Methicillin resistant bacteria had become the major problem in the infected individuals because resistant to this chemotherapeutic agent means resistance to all available beta-lactam antimicrobials. Correct evaluation in the isolation and characterization of *S. aureus* in suspected infected patients for Methicillin resistance had key role in the prescription of correct antibiotics

for the control of such type of bacteria in the hospitalized patients [19]. MRSA had also become the major causative agent of infection in the community settings [20]. The prevalence of MR *staphylococci* reported 15 to 45% of all *S. aureus* isolated from community settings and hospitals [21-23]. Now Vancomycin is the drug of choice to treat infections caused by such type of pathogenic bacteria, the emergence of Vancomycin resistant *S. aureus* (VRSA) required proper research in this context [24-25].

Antibiotic resistance study conducted in Brazilian hospital, 'Davina provedentia intensive care unit' (ICU) during 2001-2004 period showed us that most effective antibiotics were Vancomycin and Taecoplanin which were 100% effective against 104 isolated *S. aureus* [26]. Another study carried out in Bangladesh revealed that isolated MRSA showed 100% resistant to Penicillin, Cloxacillin, Oxacillin and Amoxicillin [27]. Study findings in Pakistan Institute of Medical Sciences (PIMS) hospital Islamabad showed resistance rate 92%, 60% and 58% against Ampicillin, Cephradin and Gentamicin respectively, while 38% intermediate resistance was reported against Vancomicin for *S. aureus* isolated from hospitalized and non-hospitalized patients [28]. Reports from Iraq revealed that samples collected from hospitalized patients from different age groups, the isolated strains of *S. aureus* were resistant to Penicillin G and Cefotaxime antibiotic was more effective which showed 16.66% resistance [29]. *S. aureus* isolated from hospitalized patient in Imam Khomeni hospital Teharn, antibiotic resistance towards different antibiotics showed that Vancomicin was most effective antibiotics followed by Amikacin, gentamicin, Cotrimaxazole, Tetracyclin, Cefazolin, Cephalexin, Oxacillin, Erythromycin and Penicillin were least potent [30].

1.2.1 Vincomycin resistant *S. aureus*

Vancomycin had been considered as the drug of choice for MRSA infection treatment and control, since 1980 [31]. Due to highest infection of MRSA in hospital settings caused increased prescription of Vancomycin, especially in chronic ill hospitalized patients which in turn reduced the susceptibility of MRSA to Vancomycin [32]. For example Vancomycin intermediate *S. aureus* (VISA) and Vancomycin resistant *S. aureus* (VRSA) emerged [32]. VISA first case was reported from Japanese patient with MRSA infection was found out unresponsive to Vancomycin treatment. Research findings also reported VISA from USA, Europe and Asia [33]. VRSA infection was first reported from USA in 2002 [34-35]. Since then eight other cases were also found in New York and Michigan [36-43]. VISA emerged due to the basic changes in the cell wall of bacteria and in the key metabolic pathways [44]. Two *S. aureus* strains now studied, have unexpected accelerated cell wall synthesis which makes Vancomycin trapped in the outer layer with no accessibility for target molecules [44-46]. On the other hand, VRSA may probably arise obtaining genetic materials from other bacteria called *Enterococci* [44, 47]. In vitro study revealed that *Van A* resistant genes from Vancomycin resistant *Enterococcus fecaelis* transfer to *S. aureus* [42, 47] by the process of conjugation, in at least two different clinical isolated bacteria of VRSA [40- 41, 47]. Mostly VISA and VRSA occurred due to over use of Vancomycin antibiotics usually in patients with chronic illness, such as diabetes mellitus, chronic renal failure and vascular compromised along with devitalized tissues [48].

1.3 Antibiotic resistance to ESBL producing *Escherichia coli*

Extended spectrum beta lactamases producing *E. coli* are being reported from hospitalized patients and also caused community acquired infections [49]. ESBL contain plasmid coded SHV 1, TEM 1 and TEM 2 enzymes due to which causes multi drugs resistance occurs against Cephalosporin, Penicillin and Manobactam groups of antibiotics [49]. ESBL producing *E. coli* cause failure of treatment empirically recommended but also carry resistant genes for other groups of antibiotics which decrease targeted choices for treatment [50]. Proper antibiotic prescription plays a key role in the control of ESBL producing *E. coli* infections [51]. More than 700 beta lactamases so for reported, pathogenic bacterial isolates increasing in numbers which are capable of producing multiple beta lactamases, which causes complexity in the laboratory testing [52]. ESBL are mainly produced by *E. coli*, *Oxytoca* and *Klebssiella spp.* but other Gram negative bacilli like *Proteus spp*., *P. aeroginosa, Salmonella spp.* and other *Enterobacrecia spp.* are also ESBL producers [53-57].

Antibiotic resistant towards *E. coli* reported from hospitalized patients in Tehran Iran in which 115 EBSL producing *E. coli* (60%) and *K. pneumonia* (40%) were isolated and characterized for various groups of antibiotics. Most potent antibiotics were Meropenem and Imipenem which were 100% effective against all isolates. Least effective antibiotics towards which isolated bacteria showed 100% resistance were Ampicillin, Aztreonam, Ampicillin/Sulbactam and Coamoxiclav. [58]. A research study conducted for isolation and characterization of *E. coli* in Pakistan Institute of Medical sciences (PIMS) hospital Islamabad showed that highest resistance was reported against Cefotaxime, followed by

Ciprofloxacin and Cefepime. Genetic analysis revealed in this study that class A and class C beta lactamase genes either in combination or alone were found in isolates. [59].

1.4 Antibiotic resistance to *Pseudomonas aeroginosa*

Pseudomonase aeroginosa Gram negative bacilli, belongs to *Pseudomonadaceae* family. In 1882 it was isolated from green pus [60]. *P. aeroginosa* is opportunistic pathogen causing infection in community and hospitals settings. It can cause infection in any external site of the body or organ and can be isolated from urine, sputum, ear swab, pus and even blood [61]. Center for disease control (CDC) USA and national nosocomial surveillance reported that *P. aeroginosa* is the 2nd common cause of hospital acquired infection that was 17 %, the 3rd cause of urinary tract infection that was 7%, 4th common reason of wound infection that was 8%. It is commonly isolated multi drugs resistant pathogen [60]. MDR *P. aeroginosa* widely emerged in hospitals caused serious complications in hospitalized patients [62]. Reasearch study conducted at tertiary care hospital Ahmad Abad India, revealed that 100 isolated *P. aeroginosa* showed resistance to Tobramycin followed by Gentamycin, Piperacillin, Ciprofloxacin and Ceftazidime 68%, 63%, 50%, 49% and 43% respectively. They recommended that to control the spread of MDR *P. aeroginosa* strict surveillance and supervisions policies must be implemented in the use of antibiotics [63]. Research study carried out in Tunisia Monastir regions showed that 1368 *P. aeroginosa* strains were isolated and properly identified from different hospitalized patients. Isolates of *P. aeroginosa* were mainly from pus, respiratory samples, urine and blood cultures (52.9%), (19.5 %), (10.6%) and (5%) respectively. The isolated *P. aeroginosa* showed resistant to Ticarcilline, Ceftazidime, Imipenem, Gentamicin and Amikacin up to 26.2%, 21.8%, 19.6%, 39.3%, 19.2%, and

Ciprofloxacin 21.6% respectively [64]. Mehta reported that *P. aeroginosa* isolated from pus followed by blood and other body fluids, majority of isolated strains were from Male (Male; 227, Female; 154). The isolates from blood samples showed resistance pattern towards different antibiotics were Amikacin11%, Ciprofloxacin 12% and Piperacillin 13.5%. The most effective antibiotics were Amikacin, Ciprofloaxacin and Piperacillin against isolated *P. aeroginosa* [65].

A study carried out in Hayatabad Medical Complex (HMC) Peshawar Pakistan showed us the prevalence and antimicrobial resistance pattern of *P. aeroginosa* isolated from different samples of patients from Khyber Pakhtunkhwa (KPK) admitted in various wards and the patients attended in OPD (Outpatient department). Total 314 strains of *P. aeroginosa* were isolated, in which maximum numbers (24.61%) isolated pathogens were from orthopedic ward. The infection from *P. aeroginosa* was higher in male 61.78% than female 38.22%. Antibiotic resistance pattern was higher against Ampicillin followed by Ampicillin/Sulbactam, Coamoxiclav and Ofloxacin 98.4%, 85.3%, 83.8% and 68.4% respectively. While most potent antibiotic against *P. aeroginosa* was Amikacin which showed 24% resistance pattern. It suggested proper rational utilization of antibiotics in hospital settings in such type of adverse situations [66]. Another study carried out in Jinnah Postgraduate Medical Centre (JPGMC) Karachi. Samples collected from 31 admitted patients findings revealed that *P. aeroginosa* was the prominent pathogen which caused burn wound infections. Total 44 *P. aeroginosa* strains were isolated from patients, most were multiple antibiotic resistant. Sensitivity of isolates against Imipenem was better than other antibiotics which were 77.30% and ciprofloxacin showed 54.50% sensitivity. Amikacin was effective against 30% isolated *P. aeroginosa*. The least potent

antibiotics were Aztreonam, Piperacillin, Cefutaxime, Chloramphenicol, Septran and Tobramycin [67].

1.5 Antibiotic production from microorganisms

1.5.1 Historical background

In the French microbiological literature the word antibiotic appeared before 1928 as antibiosis. The inhibitory activity between living organisms was absolutely noticed even since 1877, when antagonistic activity of aerobic bacteria against the growth of *Bacillus anthracis* was reported by Pasteur and Joubert. Selman and Waksman in 1942, introduced the world with the present narrow meaning, "a biochemical compounds extracted from microorganisms, which arrest the growth and kill other organisms in diluted form are referred as antibiotics [68]. In the decade of 1960; search for antibacterial compounds became slow down, struggles for antimycoplasmal, antitumor, antiprotozoal, antiviral and antifungal along with metabolites for antioxidants were continued [68]. Due to the misuse of antibiotics the resistant strains of bacteria emerged so the new compounds or chemically modified product of existing antibiotics had replaced the existing ones [68].

1.5.2 Why an efforts for antibiotics Search

Continuous success of researchers in the discovery of bioactive compounds as antimicrobial secondary metabolites useful for control of plants, animals and human diseases and became renewable source of compounds for important application in the field of medical and agriculture [69]. Not only in therapy against diseases, antibiotics equally useful in the development of cell and tissue culture techniques, biochemistry, molecular biology and genetic engineering [70].

The competent countries in the world for the production of new antibiotics are Japan, USA and England, which maintained leading positions. Now a days, these products of antibiotics available in various number of analog, in which minor changes had been done in earlier known antibiotics available in the market. As an outcome of rare microorganisms' isolation and characterization enhance the discovery of antibiotics extraction and evaluation of novel compounds through in-vivo assays, as a result, new antibiotics have been searched out at steady rate. Although biotransformation of antibiotics and chemical derivatives is the useful option in the scientist point of view for potential compounds production, but the search for novel antibiotics from antibiotic producing microorganisms is the more effective goal [71].

1.5.3 Concrete objectives of antibiotic research

Biotechnology offers some of the aims and objective for the development of antibiotics as compare to other multiple aims of this field. It always tried to increase the production of antibiotic during batch fermentation and further processing. Antibiotic research mostly directed towards the discovery of new compounds, due to many reasons because infections caused by many fungi, viruses and other microorganisms have no potent antibiotic. Bacteria, for example, *Pseudomonase aeroginosa* intrinsically became multi-antibiotic resistant. Due to the misuse of antibiotics and usual prescription by non-specialist physicians' antibiotic resistance emerged continuously and potentially important antibiotic toxicity to host physiology and effect on key human organs. Mostly existing available antibiotics in the market are expensive [72].

Due to the above mentioned facts it is necessary to speed up search for new antibiotics and development of optimization protocols to increase the yield of existing available antibiotic producing ability of microorganisms [72].

1.5.4 The mechanisms of action of antibiotics

Cell wall synthesis inhibitors inhibited basic steps during the formation of bacterial peptidoglycan. Some antibiotics like Penicillin, Bacitracin and Cephalosporin groups prevent cell wall synthesis. While cell membrane synthesis inhibitor antibiotics destroy or prohibited some functions of bacterial cell membranes. For example Polymyxin antibiotics extracted from *Bacillus polymyxis* prevent cell membrane formation during synthesis. Tetracycline and Chloramphenicol antibiotics prevent some basic steps in the complicated process of translation thus prevent protein synthesis. Antibiotic like Nalidixic acid and Rifamycin target DNA or RNA so their messages cannot be read after binding with these chemotherapeutic agents. While some antibiotics competitively acting as antimetabolites during operational metabolic pathways by competitively inhabiting the utilization of essential metabolites and enzymes. Antibiotics like Isoniazid and Sulfonamide drugs interfere with pyradoxal and folic acid synthesis respectively [73].

1.5.5 Functions of Soil microorganisms

Soil is the rich reservoir of microorganisms which produce secondary metabolites for their own benefits; these secondary metabolites can be exploited for the extraction of antibiotics. Soil arguably provide most favorable habitat for diverse groups of microorganisms. One gram of soil inhibited between one to ten million microorganisms majority represents bacteria and fungi. Microorganisms play important role in the soil;

these organisms enhance plant growth by increasing the nutrient and water absorption capacity of plants, and also neutralized hazardous compounds which become the major cause of pollution in the environment. Soil microbes involve in all chemical transformation and contribute actively to such types of biochemical processes. Diverse groups of soil microorganisms are change with the composition of inorganic and organic matter. Organic matter in the soil referred to as fully decompose organic constituents in the soil in which decomposed or partially decomposed animals and plants organic matter as well as soil biomass excluded [74]. Organic matter rich soil provided favorable habitat to microbial growth as compare to nutrient poor soil. Organic matter especially greatly influence the bacterial diversity, soil organic matter increased the growth of bacterial population in the soil. In many studies it is reported that bacterial diversity is hundred times greater than other microbial diversity [75]. Bacteria are the integral part of soil microbial population and not exist freely in the soil but closely attached with soil particles and organic materials even added as an isolated form [76]. In spite, microbial interaction with soil particles and organic materials they also play vital role in the decomposition of organic matter, nitrogen fixation for plant growth, biotransformation and biogas production. They operate biogeochemical cycle and increase soil fertility due to recycling of essential nutrients in the form of nitrogen, phosphorous and potassium which is necessary for the growth of plants [77]. Fungi shared more fraction of microbial biomass. Majority of plants diseases caused by fungi, moreover microorganisms which enhance plants growth are also fungi. Fungi uptake nutrients from organic materials in the soil like bacteria [78]. Rest of microbial diverse groups are actinomycetes, protozoa, algae and others also perform their microbial activities in the soil which influence the

plants growth and also the net annual primary productivity depending upon the amount of organic matter in the soil [79].

1.5.6 Some antibiotic producing microorganisms

Wide range of microorganisms produced secondary metabolites as antimicrobials activities in extended habitats. These producers inhibit aquatic environment, decomposing plants and animals etc, but large numbers of antibiotic producers are found in the soil of diverse location of the world [80].

Some antibiotic producing microorganisms are: *Bacillus licheniformic, Cephalosorium acremonium, Penicillium chrysogenum, Streptomyces antibiotics, Streptomyces griseus, Streptomyces kanamyceticus, Streptomyces fradiae, Streptomyces albinogen, Streptomyces sioyaensis, Streptomyces lavendulae, Bacillus subtillis, Streptomyces cinnamonesis, Streptomyces veneguelae, Streptomyces verticillatus, Penicillin griseofulvin, Penicillin urticae, Pseudomonas aureofaciens, Streptomyces caelestis, Streptomyces sp. X-53, Streptomyces cacaoi, Streptomyces spp. P-8648, Stretpomyces spp., Micromonospora spp., Thermophilic actinomycetes, Streptomyces spinosus, Streptomyces hygnoscopicus, Streptomyces pencetius* and *Streptomyces erythrea* which produced Bacitracin, Cephalosporin C, Penicillins, Actinomycin, Oleandomycin, Indolmycin, Streptomycin, Candicidin, Kanamycin, Neomycin, Puromycin, Siomycin, Streptothricin, Bacillin, subtillin, Monensin, Chloramphenicol, Mitomycin, Griseofulvin, Patulin, Pyrrolnitrin, Celesticetin, Echinomycin, Polyonins L and M, Viridogrisein, Novobiocin, Micromonosorin, Thermomycin, Thermocyridin, Refcin (anthracin) Spinosad, Rapamycin, Avermectin and Erythromycin [81].

1.5.7 Fungi

Diverse groups of fungi exist in soil because of its natural habitat. Fungi are the second largest fraction of soil micro flora [82]. Many bioactive compounds have been isolated from soil fungi having structural novelty, due to which now it is the important source of antibiotic and that's why out of 20 recommended medicines in the market six are from fungal source [83].

The first product penicillin was derived from fungi. This one fungal product developed a mile stone in the field of pharmaceutical research. Penicillin discovery enable people to develop new products useful in the treatment of various infectious diseases from microorganisms [84]. The discovery of penicillin in 1940 became the first useful recognized and thoroughly used antibiotic against pathogens [85-89], first human clinical trials of penicillin was conducted by late Alexander Fleming. This antibiotic was produced by *penicillium notatum* [90]. Fleming accidently discovered penicillin in 1928, and tested its invitro efficiency against many human pathogenic bacteria. The discovery of this antibiotic laid the early foundation for the development of bioactive compounds produced by microorganisms [88].

Fungi not only produced antibiotics but also important due to potential source of organic acids and enzymes [91], especially *Aspergillus* species are used in the food, pharmaceutical and paint industries [92]. In agriculture sector fungi are successfully used for the control of pest because the mechanisms of action are non-specific that's why it was applicable against broad range of insects [93]. Fungi can also convert plant lignocellulose parts into methane, hydrocarbon and carbon dioxide so methane and hydrocarbon can be used as a fuel [94].

Research study was conducted in Keffi Metropolis Nigeria, in which ten fungi were isolated from soil samples collected from ten different habitats which were active against at least one indicator strains of *E. coli, S. aureus. P. aeroginosa C. Albicans* [95].

1.6 Actinomycetes

The slow growing microorganism having high nucleic acid high G+C contents, filamentous growth Gram positive are categorized as actinomycetes. Large number of actinomycetes grows well on general purposes media which is commonly used in the microbiological laboratories, like nutrient agar, Muller Hinton agar, blood agar and trypticase soy agar. Actinomycetes composed up of large numbers of bacteria. They have important prominent role in nature and in the biogeochemical cycling [96-97].

Actinomycetes also need special media for characteristic spore formation and pigments productions. Different pigments, colors patterns for instance are shiny compact colonies of *Streptomyces spp.* on nutrient agar media can be transformed into yellow colonies white color aerial mycelium when organisms were grown on to oatmeal agar medium. The growth of actinomycetes occur from a spore during favorable environmental condition called germination or part of mycelium during exponential phase of growth which developed in to hyphae which penetrated the agar and hyphae on substrate branched form hard and tough powdery colonies on solid media. The morphology of colony depends on the nature of the medium, for example some actinomycetes colonies hyphae break down in to rods and sphere shape which form soft flexible colonies. Certain *Streptomyces* colony hyphae grow away from the substratum into air thus forming aerial mycelium [96-97].

The slow growing characteristic is obvious character of actinomycetes. With the help of light microscope we can observe the aerial mycelium even after 24 hours of growth on clear transparent agar media, while colonies visible through naked eye appear after three to four days of incubation. Fully developed growth of aerial mycelium can be observed after seven to fourteen days, some slow growing actinomycetes required 30 days of incubation period [96-97].

Strict aerobic actinomycetes carried out oxidation which is usually saprophytic, their growth restricted when oxygen supply became poor. The inoculation of actinomycetes in broth needed specialized conditions for growth. In stationary broth culture tube is commonly stopped growth, only restricted to a surface leaving behind the broth without turbidity. Broth culture need considerable shaking and aeration to grow uniformly and developed dense turbid growth. Culture tubes and inoculated flasks necessarily be kept in shaking incubator at relatively high speed that is 200 to 250 revolutions per minute (rpm), for high supply of oxygen and uniform mixing of nutrients for optimum growth [96-97].

The colony morphology of actinomycetes provide informative clues for fast identification on solid media, but just observation of isolated colony provide information which is not enough for complete identification. On the basis of morphological characteristics, identification is applicable up to generic level only in some studies. For such type of identity presence and absence of spores on the mycelium, the growth of zoospores in special types of spore vesicle formation and sporangia were considered. The motile spores' formations were more common in actinomycetes [96-97].

For long period actinomycetes can be preserved in liquid nitrogen, both spore forming and non-spore forming actinomycetes. Storage for extended period of time requires freezing at low temperature in 20% v/v glycerol at -20 C^{o} [96-97].

Spores are produced in a single unit or in aggregates of different sizes, or in the form of sporangia. Some genera of actinomycetes produce flagellated motile spores but spores are usually non-motile. Actinomycetes genera can be differentiated from each other on the basis of morphological characteristics and chemical composition of marker chemical on the cell wall, plasma membrane and cell hydro lysates. Majority of actinomycetes required oxygen for growth and survival but some needed less supply of oxygen and some cannot grow in the presence of oxygen. Actinomycetes are largely free living microorganisms, exploit vast range of environmental habitats including water and soil but some are pathogens of plants and animals. Free living actinomycetes are heterotrophic using broad range of organic compounds including complex materials as energy sources [96-97].

1.6.1Genera of actinomycetes

1.6.1.1 Nocardioform actinomycetes

This genera include large number of species which carried out filamentous growth, spore forming, aerial mycelia was produced by some species and also developed into fragments of short size. This genera was characterized by chemically heterogeneous groups of organisms including availability and non-availability of mycolic acid in the cell wall and other biochemical compounds. These comprised the following subgroups Mycolic acid-containing bacteria (Genus –Gordona, Nocardia, Rhodococcus and Tsukamurella),

Pseudonocardia and related genera (Genus –Actinobispora, Actinokineospora, Actinopolyspora, Amycolata,Pseudoamycolata, kibdelosporangium, Saccharomonospora and Saccharopolyspora), *Nocardioides, Terrabacter* and *Puomicromonospora* and related genera (Genus –*Jonesia, Oerskovia*) [96-97].

1.6.1.2 Genera with multilocular sporangia

This group of actinomycetes produced filaments which separated longitudinally and transversely septa. These included various number of sphere like components, which comprised motile groups of microorganisms like *Dermatophilus, Geodermatophilus* or non-motile *Frankia* [96-97].

1.6.1.3 Actinoplanes

The major characteristics of these genera were stable filaments formations along with minute aerial growth or no aerial growth at all, while motility of spores was the common character in *Actinoplanes*, *Ampullariella, Dactylosporangium* and *Pilimelia*. Non motile spores are produced by *Micromonospora* and Catellatospora. Cell wall composed of glycin and meso-Diaminopimelic acid (meso-DAP) while rest of the whole cell hydrolystes contained arabinose and xylose [96-97].

1.6.1.4 *Streptomycetes*

It is a diverse group and extensively studied, major features of cell wall having glycine and Lysine Diaminopimelic acid (LDAP). Consistent filaments formation and development of extensive aerial growth and spore forming ability in chain form including *Streptomyces* and *Streptoverticillium* [96-97] (Fig 2).

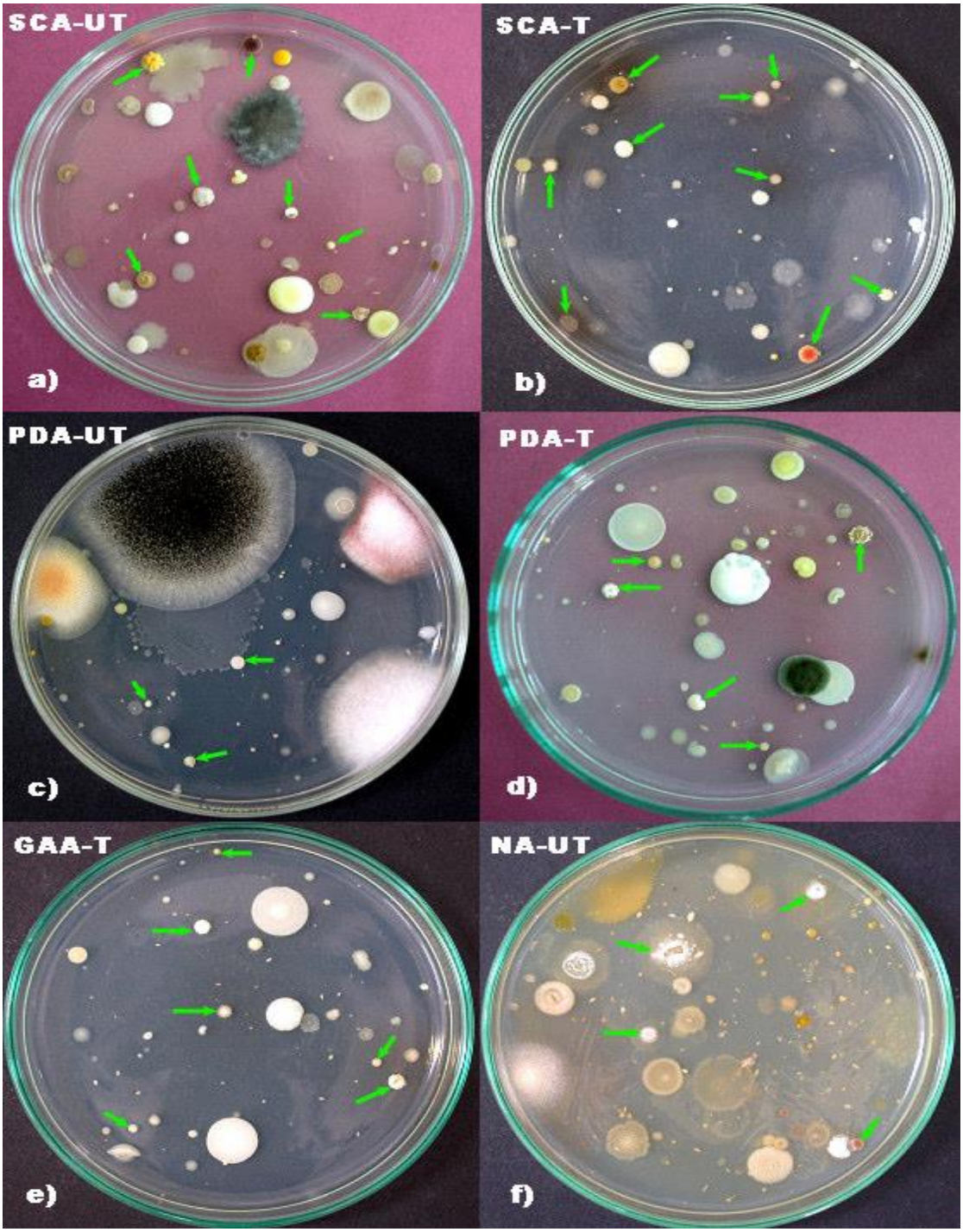

Fig 2. *Streptomyces* growth on starch casein agar media (a and b), Potato dextrose agar media (c and d), glycerol asparagine agar media (e) and nutrient agar media (f). The majority of *Streptomyces* colonies are recognizable by their spherical and wrinkled shape and diffusible pigments. *Streptomyces* colonies are shown by arrows [98].

1.6.1.5 *Maduromycetes:*

Maduramycetes synthesized stable filaments and also from aerial growth above the substratum of media. *Microbispora* produced two short chain size spores, form four spores of same chain morphology and varying numbers of spores developed by *Actinomadura*. In Other genera, sporangia is the main spores producing site with motility are *Planobispora, Planomonospora Spinillospora* and with no motility is *Streptosporangium*. Sub groups which have meso-Diaminopimelic acid (meso-DAP) in the cell walls and containe madurose in the cell hydrolysates [96-97].

1.6.1.6 *Thermomonospora*

Thermomonospora had stable filaments and developed aerial growths having single spores, *Actinosynnema, Nocardiopsis* have spores in chains, *Streptoalloteichus* have spores in sporangia. The members of this genus have meso-diaminopimelic acid (meso-DAP) in the cell wall. But these have no distinguishing sugars or amino acids in cell hydrolysates [96-97].

1.6.1.7 *Thermoactinomycetes*

This group of actinomycetes have single genus including *Thermoactinomycetes*. The members of this genus also produce filaments and aerial growth. Single spores in the form of endospores are produced on filaments and aerial growth. All species can grow at relatively high temperature that's why it is called thermophiles. The cell wall has meso-diaminopimelic acid (meso-DAP) sugars and amino acids are not found in hydro lysates of whole cell [96-97] (Fig 3).

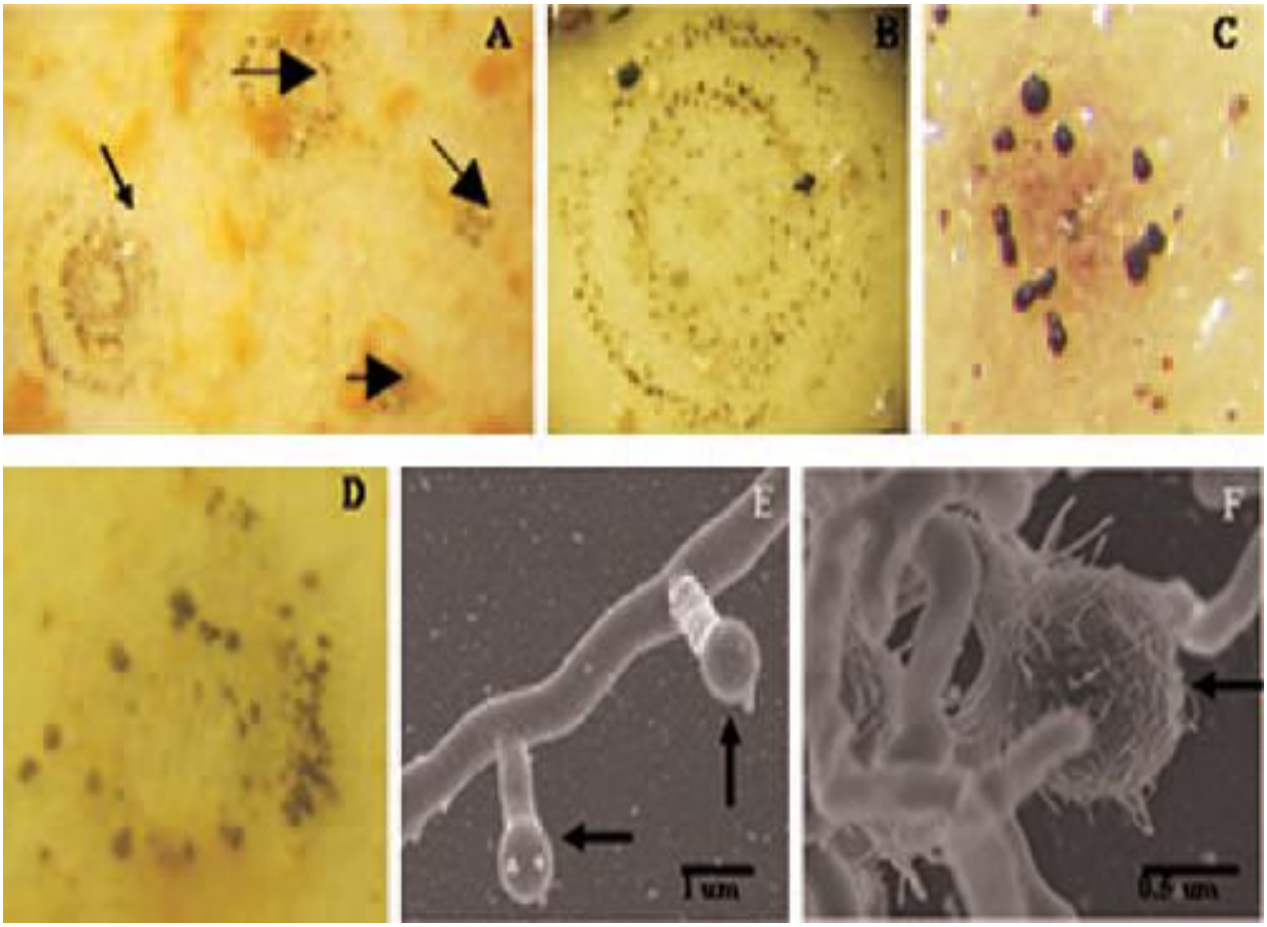

Fig 3. Spores and colonies morphology of different actinomycetes [99].

1.7 Actinomycetes as a source of antibiotics

Actinomycetes are placed in the first priority source of antibiotics. More than 5000 antibiotics have been extracted from various types of microorganisms; unfortunately only 100 are commercially available for human, animals and plants diseases for treatment. More than 60 % antibiotics are purified from the genus *Streptomycetes.* Another 15 % known antibiotics are produced by *Micromonospora, Actinomadura, Streptoverticillium* and *Thermoactinomycetes* [97].

1.7.1 Need for novel antibiotics from microorganisms

Novel antibiotics are increasingly needed for control of antibiotic resistant human pathogenic microorganisms. Extraction of novel antibiotic can be produced from natural products. After extensive research studies on actinobacteria, now it is documented source of natural producer of antibiotics. Search for novel antimicrobial bioactive compounds can be enhanced by screening and isolation of rare genera of actinomycetes. Rare actinomycetes are those actinomycetes in which the isolation frequency from their natural habitat is lower than *Streptomyces* using conventional methodologies for isolation. Many habitats are still unexploited for isolation and screening, such type of environments can be regarded as a source for the isolation of novel microorganisms which produce bioactive compounds against multidrug resistant microorganisms. These unexplored or under explored environments provide a promising diversity of microbes which need a quest for isolation and screening programs. These diverse groups of microorganisms can be used as an efficient way for combating resistant pathogenic microbial threats [100].

1.7.2 Antimicrobials from Actinomycetes

Genus *Streptomyces* is filamentous actinomycetes are well known genera of soil filamentous bacteria. *Streptomyces* produce wide range of secondary metabolites, which is most familiar for antibiotics which are at the same time use as chemotherapeutic agents and in agrochemical applications [101-103]. *Streptomyces spp*. produces two thirds of marketable antibiotics globally [104] and 60% of these commercially available antibiotics utilized for agrochemical purposes [104]. Antimicrobial resistance caused worldwide concerns, and now a considerable large numbers of chemotherapeutic agents ineffective for the control of bacterial infections [105]. The rapid increase of multi drugs resistant microbial pathogens urgently needed (on priority basis in the agrochemical industry and pharmaceutical industries) to developed trends and applicable strategies to pin point novel antibiotics through wide range screening approaches with broad spectrum potentials against large groups of pathogenic microorganisms, and may also stable against the resistant microbial enzymes [106-107].

Research studies conducted in which 134 actinomycetes were isolated from soil samples. These isolated strains were screened against large numbers of test bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*. 51 actinomycetes were active against single or multiples indicator strains, while six actinobacteria have broad spectrum activity against all tested indicator bacteria. All isolated species belongs to genus *Streptomyces,* were identified biochemically and morphologically [108].

Actinomycete, *Streptomyces spp.* isolated from marine sediment which produced antibiotic cyclo (tyrosyl-prolyl) coded as $C_{14}H_{16}N_2O_3$, which showed activities against

Gram positive and Gram negative ATCC bacteria. For antibacterial activities these indicator strains were used, *Escherichia coli ATCC 25922, Staphylococcus aureus ATCC25923, Bacillus subtilis* ATCC 66923 and *Pseudomonas aeruginosa* ATCC 27853 [109]. *Streptomyces* is common inhabitants of soil and well-studied antibiotic producers. *Streptomyces* were isolated from moula (Turkey) from various soil samples were collected from different localities. Total fifteen *Streptomyces* spp. were tested against seven microbes including antibiotic resistant *S. aureus* and *Stenotrophomonas maltophilia*. These isolated *Streptomyces* have antimicrobial activities at least against two of the tested indicator strains. This study concluded that 5 soil isolates exhibited activity very highly against (MRSA). Remaining 10 isolates were very efficient against various microorganisms, activity within the range of 30 mm zone of inhibition was observed. Gram negative bacterial strains were inhibited mostly by soil *Streptomyces* isolates. The diameter zone of inhibition was higher than 30 mm for two isolates [110].

Cirramycin-B belong to macrolid group of antibiotics was extracted and purified from *Streptomyces cyaneus.* This antibiotic has anti pathogenic effect on pathogenic microorganisms. The active fraction was extracted using ethyl acetate at the ratio of 1:1 v/v at pH 7. Cirramycin B purification was carried out using thin layer chromatography and column chromatography. The chemical formula of purified antibiotic was $C_{36}H_{59}NO_{12}$. Minimum inhibitory concentration was also determined through paper disc diffusion assay which was in the range of 1.95, 0.97, 0.97, 3.9, 3.9, 16.62, 31.25 ug/ml against *Staphylococus aureus, Micrococcus luteus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively [111]. Four new antibiotics, stretocidin A ~ D cyclic homodecapeptide, from the

Streptomyces "Tii 6071" searched out through diode array and electroscopy of HPLC methods. These antibiotics were closely related to the peptide antibiotics extracted from *Bacillus brevis* have antibacterial activities against Gram positive indicator strains of bacteria [112]. Physical pretreatment of soil samples by dry heat method in oven and the isolation media supplementation with antifungal and antibacterial agents in the quantity of Cycloheximide 80 μg/ml and Nalidixic acid 75 μg/ml. The actinomycetes recovered from soil samples were 12 CFU 10^{-6}/g in actinomycete isolation agar medium and 8 CFU 10^{-6}/g in Starch casein agar medium. Isolated actinomycetes were tested against pathogenic bacteria using two different media. Twenty two actinomycetes species were found active secondary metabolites producers against bacterial pathogens *S. aureus, B. subtilis, S. typhi* and *K. pneumonia,* in out of total 42 isolates. Thirteen isolates particularly inhibited all tested pathogens. Isolate coded "A107" was identified as *Streptomyces* specie have significant activity than all other active isolates [113]. Actinomycetes were screened out from marine environment for antibacterial activities against multiple antibiotic resistant bacteria. The indicator bacterial strains were Methicillin resistant *S. aureus* (MRSA). Total 120 actinomyctes were isolated from samples collected from sea sediments at the south east coastal area of Bengal, India. Total 10% from isolated actinomycetes were screened out had antimicrobial activity against multi drug resistance (MDR) bacteria. One strain of actinomycetes was selected for further studies. Morphological and biochemical studies were carried out and identified as *Streptomyces albofaciens*. It was Gram positive non motile spore forming and with aerial mycelium. Biochemically this actinomycetes used these carbon sources for full-fledged growth arabinose, xylose, inositol, mannitol, fructose, sucrose and raffinose, while as a

nitrogen source the isolate utilized L-asparagine, L-phenylalanine, L-histidine and L-hydroxyprolone for optimized growth. For comparison manual of determinative bacteriology was studied [114]. Penka M *et al* reported the isolation of 47 actinomycetes from the soil of Antarctica. Forty percent isolates showed antimicrobial activities against Gram positive and Gram negative bacteria. Twelve percent soil isolates revealed broad spectrum antimicrobial activities. Morphological and biochemical analysis of isolates revealed microbial diversity except strain number 23 and 29 were similar in characteristics the rest were different from each other's. The strains isolated from soil were from *Streptomyces*, *Actinomadura* and *Kitasatosporia* genera identified morphologically. Three actinomycetes were anti phytopathogenic bacteria. The antipathogenic compounds produced by these isolates may be had non polar structure and contained several active parts [115]. Another research investigation from Judith Schimana *et al.* (2000) revealed the extraction of two novel antibiotics named "simocyclone D4" and "simocyclone D8" from the mycelium extract of "*Streptomyces antibioticus*" by HPLC diode array. The isolated compounds showed broad spectrum antibacterial activities and effective on various tumor cell lines [116].

Research outcomes highlighted new members of tellocarcin group of antibiotics were isolated from fermented broth culture of actinomycetes, named as aristo-statin A and B. The antibiotic producing strain coded (TP-A 316) was rare actinomycetes belongs to *micromonospora* genera. Aristo-statin was extracted from broth culture by solvent extraction and chromatographic purification technique. This antibiotic showed narrow spectrum activity against Gram positive bacteria as well as anticancer activity [117]. Kozo Ochi reported a new methodology for increase production of antibiotic from

Streptomyces spp. by combined drug resistance mutation. The mutants were able to produce 3 fold higher antibiotic productions. Higher production was carried out among isolated *Streptomyces* resistant to streptomycin, gentamicin or rifampin. Resistant *Streptomyces* spp. grows better on solid media containing any of the mentioned drugs. Double and triple mutant strains further increase antibiotic productivity and ultimately developed a mutant strain which was able to produce 48 times more antibiotic as compare to wild type *Streptomyces spp.* Genetic analysis of streptomycin resistant mutant strains showed point mutation in the "rpsl" gene which was responsible for the synthesis of S12 ribosomal protein. Rifampin resistant mutant was found to have induced point mutation in the "rpo B" gene which carried information for the synthesis of one of the sub unites of RNA polymerase enzyme. Single, double and triple mutation revealed enhanced production of antibiotic which have Act II-ORF4 pathway a regulatory proteins was determined by western blotting technique. This is the same regulatory pathway operation in the actinorhodin productivity [118]. Marcelo reported the isolation of antibiotic producing strain belong to genus *Streptomyces* from Brazilian soil coded as Tii 6239. In the evaluation for bioactive compounds through HPLC diode array screening three bioactive compounds were find out from Tii 6239 strain. They were categorized as macrolactam group as ripromycin, ikarugamycin (already characterized) and new derivitative of Ikarugamycin, ikarugamycin epoxide. These bioactive compounds were effective against Gram positive pathogenic bacteria and also inhibited the growth of tumor cell lines [119]. JIN ZHi Hua reported in 2004 that medium optimization enhanced the antibiotic yield of spiramycin. Oil tolerant *Streptomyces* strains were screened for spiramycin production; one mutated strain *Streptomyces ambofaciens* "XC2-37"

produced 9 folds more antibiotic than the parent wild type strain *S. ambofaciens XC-1-29.* Effect of oil from soyabean and propyl alcohol on the productivity of antibiotic with *S. ambofaciens* XC 2-37 was investigated. The antibiotic production capacity of *S. ambofaciens* XC 2-37 was increased by 61.80% when 2% oil of soyabean 0.40% propyl alcohol after 24 hours incubation period. Optimum time for the addition of propyl alcohol after 24 hours incubation in batch fermentation and suitable after 20 hours incubation period in the fermentation process carried out in the fermenter. This new optimized process was designed for maximum production of spiramycin at Xinchang pharmaceutical industry [120]. Kapadnis et all reported the isolation of 312 actinobacteria from collected soil samples. Isolated actinomycetes were tested against pathogenic fungal strains. Out of 312 isolated actinomycetes, 22% were potent against tested fungi. One most potent soil isolate with highest activities against fungal strains was selected for further research investigations which was effective against mold and yeast. Optimization was carried out for divers' parameters and identified morphologically and biochemically as *Streptomyces*. Maximum production of antibiotic was obtained in the late log phase of growth which continued constantly up to stationary phase. The secondary metabolites were extracellular in nature [121]. Jain et al investigated the isolation of *Streptomyces purpeofuscus* coded CM 1261from composted soil have excellent activity against four human pathogenic fungi. The bioactive compound was heptaene group of polyen antibiotic [122]. Shu-Wei Yang *et al* find out novel microbial secondary metabolite named "sch 725424-1" was extracted from broth culture of *Kitasatospora spp*. The isolated compound revealed best antibacterial activity against *S. aureus* with MIC range of 1-2 µg/ml and also revealed antifungal activity against *S. cervisiae* [123]. Noemi Antal

et al investigated the isolation of new compound known as retymicin along with galtamycin B and Saquayamycin Z which are the new members of galtamycin and Saquayamycin families, new compound isolated from strain Tii (6368) belong to *Micromonospora* genera isolated from Romanian soil. These compounds revealed cytotoxic activity to different human tumor cell lines in-vitro, while saquayamycin Z also have narrow spectrum activity against Gram positive bacteria [124].

Another study revealed the effect of various carbon and nitrogen sources optimized for the production of non polyenic macrolide antimicrobials produced by *Streptomyces hygroscopicus* 111-8. Instead of the addition of glucose substituted with glycerol or lactose increased antibiotic production. Best source of nitrogen for maximum production of antibiotic was ammonium succinate. Divalent ions like $Mn^{2+,}$ $Cu^{+2,}$ Fe^{2+} increased antibiotic biosynthesis from AK-111-81. These findings became helpful for the development of new fermentation medium showed six times increased production of antibiotic under investigation in this study as compare with the basal medium [125].

1.8 Aims and objectives

Aims and objectives of the study are:

1. Isolation and characterization of soil microorganisms and hospitalized human pathogenic bacteria in KPK.
2. Screening of antibiotic producing microorganisms from soil isolates.
3. Optimization of various parameters for maximum production of antibiotic.
4. Extraction of antibiotics from soil isolates and checking of antibiotic extracts against selected human pathogenic microorganisms.

This research work was divided in to three phases; in the first phase, hospitalized human pathogenic bacteria were isolated, identified and characterized for various available antibiotics to

know about resistance pattern. In the second phase actinomycetes and fungi were isolated from soil samples and characterized. In the third phase of research study isolated actinomycetes and fungi were screened out for the antimicrobial activities against isolated human pathogenic bacteria and ATCC bacterial strains. Potent isolated antibiotic producing microorganism was selected for optimization and extraction of antibiotics and determination of minimum inhibitory concentration (MIC) of extracted antibiotic against multi drugs resistant (MDR) pathogenic bacteria.

Materials and Methods

For this research study ethical permission has been granted by the Research Ethical Committee University of Peshawar. Annexure 1.

2.1 Hospital selection for sample collection

Lady Rading Hospital (LRH) Peshawar was selected for specimen collection from hospitalized patients. Samples were collected from hospitalized patients in different wards. This hospital is also known as Gernaly huspatal (hospital) because it is big hospital in the province of Khyber Pakhtunkhwa (KPK), which provides curative services to the ailing community of this area and also to the afghan refugees residing in the province. Not only human population living near Peshawar get medical facilities from this hospital but people living far away in different districts of KPK is referred to this hospital by district headquarters (DHQs) hospitals of all districts in case of serious conditions of patients. It is tertiary care hospital and providing medical facilities in wide areas of clinical fields. LRH Peshawar is also a teaching hospital, providing training facilities to doctors and paramedical staff. LRH comprises of Medical A, Medical B, Medical C, Children A, Children B, Cardiology, Neurology, Surgical A, Surgical B, Surgical C, Gynae A, Gynae B, Gynae C, Optelmology and ENT (Ear Nose Throat) wards. Bio data of the admitted patients in these wards showed that they belong to different districts of KPK.

2.2 Sample and bio data collection from hospitalized Patients

Specimens were collected from different wards visited during the period of April 2010 to March 2011. Hospitalized patients were interviewed for age groups; gender, ethnic back ground, and history of previously used any antibiotic at the time of specimen collection.

Specimens like urine, and blood were collected in sterilized bottles while sterilized cotton swabs were used for pus, sputum, ear swab and cerebrospinal fluid (CSF) samples. Collected specimens were immediately transported to the laboratory for further processing.

2.3 Isolation of human pathogenic bacteria

Culture media prepared for the isolation and growth of various types of pathogenic bacteria, were: Mc Conkey agar, Eosin Methylene blue (EMB) agar, Blood agar, CLED (Cystine lactose electrolyte deficient), MSA (Manitol salt agar), Kliger iron agar (KIA), and Muller Hinton agar medium. All media were prepared in the distilled water and autoclaved for sterilization at 121 ^{0}C, 15 psi pressure for 15 minutes. Sterilized media were allowed at 50 ^{0}C and were poured in to sterilized petri plates for solidification. Sterility of the media was checked by incubating the plates at 37 ^{0}C for 24 hours. Samples were streaked on prepared sterilized media using commercially available sterilized cotton swabs. All plates were incubated at 37 ^{o}C and checked for growth of pathogenic bacteria in 24 to 72 hours incubation time. After incubation period growth of pathogens were observed.

Morphological study of the isolated bacterial colonies was carried out including colony color, size of colony, and colony shape on different media. Gram property using Gram staining kit was carried out after sub culturing for pure culture isolation.

2.4 Biochemical tests for identification of clinical isolates

For specie level identification of the isolates different biochemical tests performed were; DNase, coagulase, catalase, Urease, Triple Sugar Iron, Nitrate reduction, Methyl Red Voges Proskauer (MR-VP), Urease, voges prokauser, beta galactosidase, lactose, mannitol, glucose, sucrose utilizations, Oxidase, citrate, indole, Lysine decarboxylase (LDC) and H_2S production [126].

2.4.1 Nitrate reduction test

2.4.1.2 Required materials

Fresh culture of test pathogenic bacteria, trypticase nitrate broth, Sulfanilic acid (solution A), alpha naphthylamine (solution B), zinc in powder form, Bunsen burner, inoculating loop and permanent glassware marking pen.

2.4.1.2 Principle

Nitrate reduction is carried out by pathogenic bacteria in the absence of molecular oxygen. In these bacteria energy is produced during anaerobic respiration in which the cell machinery utilized inorganic materials such as nitrates and sulphates to supply oxygen which is further utilized for final hydrogen acceptor during energy formation. Bacteria can also subsequently convert nitrate to ammonia and molecular nitrogen through enzymatic pathways. The analysis of nitrate reduction required cultivation of test bacteria in nitrate broth medium. This medium contained potassium nitrate (0.1%) as a nitrate source. The medium is prepared in semisolid form by addition of 0.1 % agar. Semisolid media decrease the supply of oxygen diffusion in to the medium which is

necessary for nitrate reduction. After incubation period solution A and solution B were added to culture. Appearance of cherry red color indicates nitrate reduction by particular bacteria.

2.4.1.3 Procedure

Each test pathogenic bacteria was inoculated in to culture tubes containing Trypticase nitrate broth media in aseptic environmental condition. All culture tubes were plug tightly and incubated at 37 ^{o}C for 24 hours. After incubation period solution A and solution B were added. Cherry red color was observed for positive results.

2.5 Catalase test

2.5.1 Requirements

Fresh culture of test pathogenic bacteria, Trypticase Soy Agar (TSA) slants, 3% Hydrogen peroxide, Bunsen burner, Inoculating loop, clean slides and permanent marking pen.

2.5.1.2 Principle

During aerobic respiration bacteria produced hydrogen peroxide, in some cases superoxide. These substances are extremely toxic and cause death of bacteria. Bacteria degrade these toxic substances through enzyme production called catalase and superoxide dismutase. Strict anaerobic bacteria cannot produce catalase or superoxide dismutase enzyme. Catalase production can be determined by adding two drops of substrate H_2O_2 in to the center of slide and then mixing of test bacteria with the substrate using inoculating

loop. If bubbles are produced it indicates positive catalase test. Production of catalase enzyme, degrade the substrate to produce oxygen.

2.5.1.3 Procedure

Clean glass slides were labeled for each test pathogenic bacteria and two drops of 3% H_2O_2 was added at the center of slide. Fresh culture of rich inoculum from TSA slants was taken and mixed with H_2O_2 using inoculation wire loop. Bubble formation was observed within 10 seconds for catalase positive bacteria, while no bubbles formation after 10 seconds was characterized as catalase negative.

2.6 Urease test

2.6.1 Requirements

Christensen's urea broth, screw capped culture tubes and tests microorganisms

2.6.1.2 Principle

Christensen's urea broth medium contains urea and phenol red an indicator for color change from pink to red by production of urease enzyme by bacterial species. Hydrolysis of urea yield ammonia and carbon dioxide. Ammonia production changed the pH of medium and became alkaline. The variations in pH alter the chemical properties of phenol red that is pink red at alkaline pH and yellow orange at neutral/acidic pH. Negative results showed no hydrolysis and the color of medium was Yellow-orange.

2.6.1.3 Procedure

Isolated bacteria were inoculated in to the sterilized 3 ml of Christensen's urea broth incubated at 37 ^{0}C for 18-24 hours. Change in color of the media was observed that's only pink-red coloration for positive isolates.

2.7 Oxidase test

2.7.1 Requirements

Oxidase reagent, clean petri plate, sterilized tooth picks, piece of filter paper and test pathogenic bacteria

2.7.1.1 Principle

Pathogenic bacteria are able to produce oxidase enzyme extracellularly which oxidized the substrate phenylenediamine producing a deep purple color intermediate. Tests pathogenic microorganisms capable of producing oxidase enzyme oxidized phenylenediamine in the oxidase reagent and purple color appeared.

2.7.1.2 Procedure

Filter paper was placed in a sterilized clean petri dish and 3 to 4 drops of oxidase reagents was added. Fresh culture of tests pathogenic bacteria was transferred to the piece of filter paper. Outcomes were observed for color change to blue purple within 30 seconds. Positive oxidase test showed blue purple color. Negative oxidase test revealed no blue purple color appearance. For positive control *P. aeroginosa* was tested at the same time while for negative control *E. coli* was tested.

2.8 Indole test

2.8.1 Principle

Bacterial culture are grown on a medium containing tryptophan and Kovacs reagent that's 4 (p)-dimethyl-amino-benzaldehyde as used as indicator which reacts with indoel produced by *Enterobacteria spp.* imparting red color.

2.8.1.2 Types of indole test

Indole test can be performed in the following ways

1- Indole test adding tryptophan water and kovacs reagent

2- As a dual beta glucorinidase indole test adding indole identification tablets and kovacs reagents. It is especially useful for the identification of *E. coli.*

3- As a collective lysine decarboxylase indole test using LDC indole identification tablets. LDC was useful for the identification of salmonella and shigellae species.

2.8.1.3 Procedure

2.8.1.4 Indole test using tryptophan water

Test human pathogenic bacteria were inoculated in to culture tubes containing 3 ml of tryptophan water. The inoculated culture tubes were incubated at 37 ^{0}C for 24 to 48 hrs. After incubation period 0.5 ml of kovacs reagent was added and examined for color change to red on the surface within 10 to 12 minutes. Color changed at the surface layer to red revealed positive outcomes, while lack of red surface layer showed negative results.

2.8.1.5 Indole test using indole tablet

Dense suspension of the test pathogenic bacteria was prepared in 0.25 ml saline in test tube, and then indole tablets were added and the test tubes were closed tightly. All test

tubes were incubated at 37 ^{0}C for 18 to 24 hrs. After incubation period observed the beta-glucuronidase reaction. Then a kovacs reagent was added after 5 minutes indole reaction was observed and examined the color of the surface layer. Yellow color observation showed positive outcomes while no color observation revealed negative results.

2.8.1.6 LDC/Indole tablet

Test pathogenic bacteria were inoculated in 0.25 ml saline solution in a test tube. After adding LDC/indole tablets, added 3 drops of paraffin oil which provided oily layer for anaerobic conditions necessary for LDC reaction, and then closed the tubes tightly. Incubated at 37 ^{o}C for 24 hours. First of all LDC reaction was observed. For indole reaction 3 drops of kovac's reagent was added wait for 3 to 5 minutes and then observed the outcomes for the changed color of surface layer. Blue violet color indicated positive LDC test while yellow green color revealed negative LDC test. Red color of surface layer showed positive indole test while yellow surface layer indicated negative indole test.

2.9 Methyl red test

2.9.1 Requirements

Fresh Soy broth culture of test pathogenic bacteria, MR-VP broth media, methyl red indicator, Bunsen burner, inoculating loop and test tubes along with marking pencil

2.9.1.2 Principle

All enteric microorganisms utilize glucose for energy production. They produced organic acids from the fermentation of glucose. This test has immense importance in the differentiation of *E. coli* from *E. aerogenes.* The production of acids is determined by adding pH indicator methyl red in culture after incubation.

2.9.1.3 Procedure

Each pathogenic bacteria was inoculated in to labeled test tubes containing MR-VP broth media using inoculating loop in aseptic environment. All cultured tubes were incubated at 37 ^{0}C for 24 to 48 hours. Results were observed after incubation period in the form of changed color of the medium.

2.10 Voges Proskauer test

2.10.1 Requirments

Barritts reagent, Bunsen burner, inoculating loop, MR-VP broth media, permanent glass ware marking pencil and 24 hours fresh culture of test human pathogenic bacteria.

2.10.1.2 Procedure

Each human pathogenic bacterium was inoculated into already prepared media in the labeled test tubes. One test tube contained media served as control. All cultured tubes were incubated at 37 ^{o}C for 24 to 48 hours. Changed color of medium was observed for positive results while no color changed for negative results.

2.11 Citrate utilization test

2.11.1 Requirements

Simmons citrate agar slants, test human pathogenic bacteria inoculating needle, permanent marking pencil and Bunsen burner.

2.11.1.2 Principle

In this test some bacterial pathogens used citrate as only source of carbon

2.11.1.3 Procedure

Simmon's citrate agar media was prepared and poured in to the culture tube for the preparation of slants. Sterility of the prepared slants was confirmed by incubating for 24

hours at 37 ^{o}C. Tests bacteria were stabbed into the butt of slants. Incubated for 24 to 48 hours at 37 ^{o}C. After incubation period the slants were observed for blue color in the medium. Color changed after incubation period showed positive citrate utilization while no change in color of the medium revealed negative results.

2.12 Coagulase test

2.12.1 Requirements

EDTA, Rabbit plasma and test pathogenic bacteria

2.12.1.2 Principle

This test applied for the identification of pathogenic bacteria which were capable of producing coagulase enzyme. Coagulase enzyme converts fibrinogen to fibrin through activation of coagulase reacting factor available in rabbit plasma. Coagulase secreted by bacteria was detected by clotting reaction in the test tube. In the fast slide test bounded coagulase enzyme with bacteria determined due to clumping of tested bacterial cells.

2.12.1.3 Slide test procedure

A drop of distilled water was added at the center of two slides using 1 ml micropipette. Two thick suspensions were prepared by mixing the previously Gram stained fresh colonies of test bacteria cultured on nutrient agar media. Test pathogens were inoculated in to one slide suspension and mixed slowly and observed for clumping within 10 seconds. Second slide was used only for comparison to differentiate any granular appearance of test bacteria from true coagulase clumping. Clumping appeared within 10 seconds showed positive results while no clumping revealed negative outcomes. *E. coli* was used as a negative control while *S. aureus* was used as positive control.

2.12.1.4 Test tube method procedure

Three test tubes were used for test tube coagulase test and labeled as T for test, P for positive control and N for negative control by using permanent glassware marking pencil. 0.2 ml plasma was added to each test tube and then 0.8 ml nutrient broth was added to tube labeled T and inoculated the test pathogenic bacteria while 0.8 ml nutrient broth media was added to positive control tube which was *S. aureus* inoculated. 0.8 ml nutrient broth was added to test tube labeled N for negative control. Each tube shakes well for proper mixing and incubated for at least 1 to 3 hours at 37 ^{o}C. Results were observed for clotting after one hour incubation period. For satisfactory confirmation the test tubes were placed at room temperature for 24 hours and then examined the outcomes. Clotted tube contents showed positive results while no fibrin clots revealed negative outcomes. The results were compared with positive and negative controls.

2.13 DNAase test

2.13.1 Requirements

DNAase agar plate and Hydrochloric acid (HCl)

2.13.1.2 Principle

Enzyme DNase reacts with DNA and hydrolyzed it. Bacterial species produced DNAase enzyme with hydrolysis of DNA in the surrounding media. Addition of HCl after incubation causes precipitation of unhydrolysed DNA, while clear zone around cultures indicated DNA hydrolysis.

2.13.1.3 Procedure

Petri plate was divided in to the required portions by marking the underside of plate using permanent glassware pencil. Test pathogenic bacteria were spotted in to the appropriately designated portion of the plate, incubated for 24 hours at after 24 hours at 37 ^{o}C. Hydrochloric acid was poured on the whole surface of medium. Outcomes were recorded after 5 minutes. Clear zone around the colonies revealed positive outcomes.

2.14 KIA (Kligler iron agar) media, slants preparation for identification of pathogens

2.14.1 Principle

Inoculation to KIA media depends on lactose fermentation, D- glucose fermentation and the hydrogen sulphide production. Color changed to yellow at the butt indicated acid production and pink color appearance at the slope revealed glucose fermentation. Gas production due to glucose fermentation was obvious from cracks and bubbles development in the medium. The fermentation of lactose in the medium indicated yellow color slope and butt. While red-pink slope and butt revealed no fermentation of glucose and lactose. Around the stabbed line Black color revealed Hydrogen sulphide production.

2.14.2 Procedure

KIA media was weight carefully and mixed in distilled water and dispensed in to 6 ml of it in large sized test tubes, following sterilization the medium was allowed to solidify at slanted position with slope upper and butt at the bottom. Test pathogenic bacteria were inoculated using inoculation needle in the butt region following surface inoculation on the slant slope. Slants were incubated at 37 ^{o}C for 24 hours. After incubation results were observed.

2.15 Antibiotic sensitivity assay

Antibiotic sensitivity assay was conducted through Kirby-Bauer method [126]. Twenty four hours fresh culture of pathogenic bacteria was inoculated into 5 ml nutrient broth and then incubated at 37 ^{o}C for 24 to 48 hours, then turbidity of indicator strains were adjusted with Mc Farland solution adding 0.89% saline solution in culture tube of indicator pathogenic strain. Uniform lawns were prepared on Muller Hinton agar media for each pathogen using sterile cotton swabs. Antibiotic discs were gently placed on the prepared lawn at equal distance using sterile forceps. Incubated for 24 to 48 hours at 37 ^{o}C, after incubation, zone of inhibition was measured for determination of sensitivity towards different antibiotics.

2.16 Production of extended spectrum beta lactamases (ESBL)

2.16.1 Combined disc method

Muller Hinton Agar plates were prepared and selected pathogenic bacterial strains were inoculated. Turbidity of pathogens was adjusted with 0.5 Mc Farland solution to prepared lawn on sterilized solidified media. Commercially available antibiotic discs ceftazidime, cefotaxime 30 μg each, with and without clavulanic acid, was recommended as ESBL producers [127].

2.17 Area Selection for soil sampling

Suitable area selection plays an important role in the outcomes of research; research study conducted here depends upon the availability of health care facilities, trained physicians, ratio of literacy in the human population and previous studies regarding

antibiotic resistance and nature of soil and previous research on screening of soil for isolation of antibiotic producing microorganisms.

Keeping in view the above facts KPK is the second poorest province of Pakistan where people have less access for basic health care facilities and low literacy rate. Therefore, the microbial resistance has increases in the population alarmingly due to the misuse of antibiotics.

The soil of KPK has not been thoroughly investigated for screening of antibiotic producing microorganisms. However selected area presents a valuable habitats for isolation of antibiotic producing microorganisms and for evaluation of novel isolates. They may possess activities against antibiotic resistant pathogenic bacteria as a preliminary controlling effort for resistance as well as to develop trends in the pharmaceutical research within Pakistan.

The overall area of KPK province is about 74521 square kilometers and comprises 25 districts (Fig 4). According to the 1998 censuses, the population of this province was about 17 million, in which 48 % are females and 52 % are male. Among this population the majority are Pashtun which is afghan ethnic group. Besides the native population, 1.5 million afghan refugees are also living in this province since 1980.

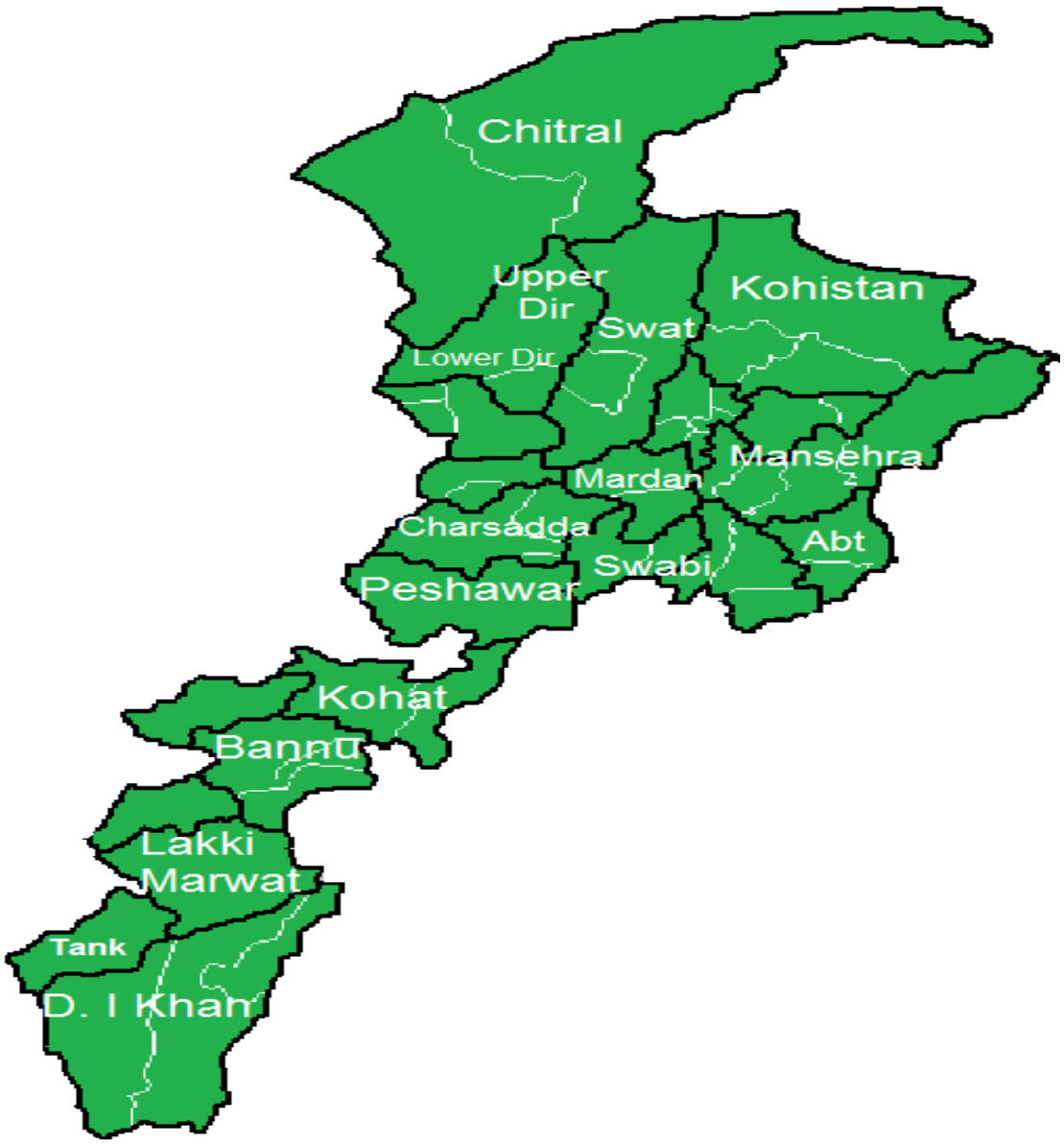

Fig 4. Districts and boarders of KPK

2.17.1 Collection of soil samples from various habitats

Soil samples were collected from diverse localities of KPK. Total 80 soil samples were collected from at 1.5 to 2 kilometer distance at every single locality of Swat valley, Bunir, Mansehra, Dir upper and Dir lower districts of Khyber Pakhtunkhwa (KPK) during April 2011 to September 2011. All different localities in these valleys were Miaadam hilly areas, Pizagat, Madian and Matta plain agricultural land of Swat valley while Peer baba ghazi khanay plain agriculture land and Peer abhy plain areas, Daggar hilly areas forest, Cheena asad abad bar kalay nonagricultural land and Gagarra nonagricultural land of Bunir valley. From district Mansehra Galiate mountainous forests were targeted for samples collection. Different localities of Dir upper and lower were Baghhdush, shahikhel, Bandagi, Noorakhel, Chakdara, Barawal, Warri, Chapar and Kalakot. Samples were collected from agricultural and non-agricultural land in plain areas of Swat and Bunir valleys while from mountainous areas of Swat, Bunir, Dir and Galiat soil samples were collected from forests. Within the forest novel habitats were targeted for collection of soil. Sampling sites were determined by GPS (Global positioning system) coordinate. Soil samples were taken from surface up to 20 cm depth using measuring scale. Samples were put in the air tight polythene bags and properly labeled. Samples were collected from Samples were kept in sterile polythen bags, immediately brought to the laboratory and dried under shade for seven days [128], crushed and sieved for isolation of actinomycetes, stored at 4 ^{0}C till isolation procedures.

Table 1. Collection of soil samples from various localities in KPK.

S.N	District Mansehra	S.N	District Swat	S.N	District Bunir	S.N	District Dir lower	S.N	District Dir upper
1	Bara Gali UoP campus n=6	1	Miaadam n=3	1	Peer baba Ghazi khanay n=1	1	Baghhdush khel n=4	1	Baraawal banda n=7
2	Bara Gali to Natia gali track n=6	2	Matta n=2	2	Peer abhy n=1	2	Chakdara n=6	2	Warri n=3
3	Bara Gali UoP campus track n=3	3	Madian n=2	3	Daggar n= 1	3	Shahikhel n=5	3	Kalakot n=2
4	Kala bagh to Natia Gali track n=4	4	Piza Gaatt n=1	4	Chena asad abad bar kallay n=2	4	Bandagi n=3	4	Cappar n=4
5	Donga Gali n=3			5	Gagarra n=2	5	Noorakhel n=4		
6	Ayubia n=2								
7	Nattia Gali urban n= 3								

Key: n=number of soil samples collected at the given ecosystem

2.18 Isolation of actinomycetes from soil

Soil samples were serially diluted up to 10^{-4} to 10^{-5}, randomly selected diluted samples were used for plating on actinomycetes isolation agar (AIA) and nutrient agar (NA) media containing Nystatin and cycloheximide 50 µg/ml as antifungal and antibacterial agents. One gram of each soil samples sieved and pretreated with 0.1 gram $CaCo_3$ was added to 99 ml sterile distilled water and kept in orbital shaker at 150 rpm for half hour to mix and suspend completely. Serially diluted samples were then plated in duplicate using actinomycetes isolation agar media. Another soil sample was plated on nutrient agar media in duplicate. All plates were incubated at 28-30 ^{o}C for 2 to 3 weeks. During incubation period growth of actinomycetes were observed intermittently. Selected colonies of actinomycetes were sub cultured in actinomycetes isolation agar (AIA) media and nutrient agar media for screening of antibiotic producing actinobacteria.

2.18.1 Indicator bacterial strains for preliminary screening of active isolates

American Type Cell Culture (ATCC) bacteria was provided by Pakistan Council of Science and Industrial Research (PCSIR) laboratories Peshawar including *Pseudomonas aeroginosa* ATCC# 27853, *Staphylococus aureus* ATCC# (25923), *Escherichia coli* ATCC# (25922), *Enterococcus faecalus* ATCC# (29212), *Klebsiella pneumoniae* ATCC# (700603) and *Micrococcus luteus* ATCC # (10240). Pathogenic Gram positive and Gram negative bacteria isolated in the first phase of research. Bacterial strains were cultured in culture tube slants and preserved at 4 ^{o}C till use.

2.19 Taxonomic characteristics

Most potent actinomycetes were identified on the basis of colony morphology, different morphological characteristics on ISP media, light microscopy at 100 X, and biochemical tests. Morphological characteristics of isolates were determined as proposed in the Berges manual of determinative bacteriology and biochemical tests were carried out according to the methods of [129].

2.19.1 Morphological identification of actinomycetes

Isolated actinomycetes were characterized for cultural and morphological properties. Soil isolates were cultured in to the international *Streptomyces* project media. The media were prepared during ongoing methodology step by step. Media was autoclaved for 15 minutes at 121 ^{o}C and 15 psi pressure for sterilization. Sterile media was poured in to sterilized petri plates for solidification. Selected soil isolates were streaked on to the surface of solidified media for growth and incubated at 28 ^{o}C for one week. Colony characterizations such as types of aerial hyphae, and vegetative hyphae growth, characteristics of fragments and development of spores were studied morphologically.

2.19.1.2 Microscopy of soil isolates

Gram staining procedure was carried out before microscopy. A drop of water was placed at the center of clean glass slide and selected soil isolates were transferred to the slide aseptically and mixed well by sterilized wire loop for the preparation of smear. Then it was allowed to dry in the air, heat fixed by passing twice through the Bunsen burner. Fixed smear was flooded with crystal violet for one minute. The slide was washed under

running tape water to remove unbound crystal violet. After drying, the mordant Gram iodine was added on to smear for one minute and then washed under running tape water. Ethyl alcohol 95 % was added drop by drop as a decolorizing agent. Safranin was used as a counter stain for 45 seconds and washed. The slides were dried with blotting paper and observed under microscope at 100 X using immersion oil.

2.20 Biochemical Tests for identification of actinomycetes

Biochemical identification tests were performed including formation of melanin pigments, reduction of nitrate, Proteolytic properties/milk coagulation, liquefaction of gelatin, hydrolysis of starch, assimilation of carbon sources, production of acid and production of H_2S.

2.20.1Test for melanin pigments

The purpose of this test is to examine the isolated actinomycetes for the production of pigments and its secretion in to the media. Secretion of pigments in to the media is desirable property of actinomycetes. Large number of actinomycetes isolated from soil has the ability of secreting of melanin pigments in to medium known as Waksman medium 42. Variety of pigments also depends upon the composition of chemically defined media, which is used for taxonomical identification on the basis of whether pigments produced is in aerial mycelium or vegetative parts or developed near the colony or secreted in to the whole medium. Pigments produced by variety of actinomycetes have great natural diversity. Pigments production depends on many factors including useable medium contents, growth requirements factors, age of inoculum and culture conditions. Thus when definite composition of known concentrations of reagents were used and

required cultural conditions were used pigments production is easily determinative characteristics of actinomycetes.

Clean test tubes with screw capped were used for the preparation of Waksman media, sterilized in autoclave for 15 minutes at 121 ^{0}C and 15 psi pressure. Sterility of the media was checked after incubation. Isolated actinomycetes were streaked by inoculating the actinomycetes in to media. The inoculated culture tubes were incubated for 4 days at 28 ^{0}C. Incubated actinomycetes were observed for pigments production intermittently up-to 4 days.

2.20.1.2 Nitrate reduction test

Nitrate can be reduced by variety of actinomycetes. Due to nitrate reducing ability of actinomycetes, actinomycetes are divided in to the following groups.

1- Actinomycetes which carried out minimum reduction of nitrate or no reduction ability.
2- Actinomycetes which carried out optimum reduction
3- Actinomycetes which carried out maximum reduction

Keeping in view the above mentioned properties of actinomycetes the soil isolates were characterized for nitrate reduction properties using nitrate broth. Nitrate broth was prepared and sterilized. Nitrate broth (10 ml) was used for each under investigation actinomycetes. Actinomycetes were inoculated under sterile environment. The inoculated tubes were incubated at 28 ^{0}C for 120 hrs. After incubation period, the nitrate reducing ability of actinomycetes was determined by adding naphthalene solution (A) and

sulfonilic acid solution (B). Two drops each of reagent A and reagent B was added. Positive outcomes revealed pink color.

2.20.1.3 Test for proteolytic activity

2.20.1.4 Coagulation of milk

Pasteurized skimmed milk was utilized for the determination of proteolytic activities by isolated soil actinomycetes. Actinomycetes positive for proteolytic activities carried out digestion of milk protein. Test tubes containing pasteurized skimmed milk were inoculated aseptically and incubated at 35 ^{o}C for 24 to 48 hours. Litmus paper reduction and changed color of the medium after incubation period were considered for positive outcomes. Color changed due to the digestion of milk protein and changed the pH of the medium.

2.20.1.5 Liquefaction of gelatin

Gelatin also acts as solidifying agent. Microorganisms grew in the medium secrete enzyme which liquefied the nutrient gelatin medium. NGA medium slants were prepared and isolates were aseptically inoculated in to the tubes of slants by stabbing procedure. Test tubes cultures were placed at 25 ^{o}C for 8 to 10 days. Secreted enzymes of soil isolates can hydrolyze gelatin. The growth of most soil microorganisms can be supported by using nutrient gelatin media.

2.20.1.6 Test for starch hydrolysis

Starch hydrolysis was studied by inoculating the soil isolates in to the starch agar medium. Actinomycetes isolated from soil if capable for enzyme production will hydrolyze starch easily. Slants of sterilized media were prepared and soil isolates were inoculated in the germ free environment. Cultured slants were incubated at 28 ^{0}C for 7 to

8 days. Iodine solution was utilized for the observation of starch hydrolyzing activity of capable isolates.

2.20.1.7 Test for carbohydrate assimilation

Actinomycetes identification also depends upon the assimilation of source carbohydrates used for their own essential requirements. This property of actinomycetes can be exploited for taxonomic grouping of actinomycetes. Carbon utilization agar medium was used for the carbon source assimilation. This medium contained bromocresole purple dye as an both broth and agar. Successful assimilation of carbon revealed medium color change from purple to yellow. Medium (ISP 9) was prepared 1 ml broth culture of soil isolates were transferred to ISP 9 agar media, transferred in to sterile petri plates. After solidification disc having 3% different carbon sources such as dextrose, lactose, maltose, sucrose and starch were placed gently on the surface of solid medium using sterilized forcefs. The plates were incubated at 28 ^{o}C for 24 hours. Occurrence of growth near or around the discs and color change from purple to yellow indicated the utilization of different sources of carbohydrates.

2.20.1.8 Test for acid production

Glucose nutrient broth media was prepared aseptically. Isolated actinomycetes were inoculated and incubated at 28 ^{0}C for two weeks. Every day the cultures were observed for change in color of the medium. Acid production was revealed by change in color from blue to yellow.

2.20.1.9 Test for Hydrogen sulphide production

Hydrogen sulphide gas is produced by many actinomycetes by fermenting the protein. Hydrogen sulphide production medium contained peptone ingredient cysteine. By the

action of secreted enzyme cysteine desulfrase cysteine loses sulfur which reacts with hydrogen ion in water to produce H_2S. Slants of hydrogen sulphide media were prepared and inoculated with soil isolated microorganism. Slants were incubated for 96 hours at 37 ^{0}C. Rotten egg smell was checked after incubation, and color of the medium was changed to black.

2.21 Collection of soil samples for isolation of fungi

Soil samples were collected from three different hospitals of Peshawar. Soil samples were targeted from such places in the hospitals which were contaminated with hospital disposable materials such as cotton, syringes and plastics. Total 33 soil samples were collected. All soil samples were taken from surface to 10 cm depth in polythene bags. Collected soil samples were brought to the laboratory for isolation of fungi.

2.21.1 Isolation of Fungi

Soil samples collected from different teaching hospitals were serially diluted up to 10^{-5}. One ml of diluted samples was uniformly spread on potato dextrose agar and Sabroud dextrose agar media by L shaped glass rod. Inoculated plates were incubated for 72 to 120 hours at 28 ^{0}C. Growth of fungi was observed, picked up the fungi and subcultured on PDA and SDA media to purify (Table 2).

Table 2. Fungal strains isolated from soil samples collected from various hospitals of Peshawar.

Hospitals	Number of soil samples	Number of fungi isolated
Khyber Teaching Hospital	9	RM 75, RM76, RM77
Lady Rading Hospital	13	RM 78, RM79, RM80, RM81
Hayatabad Medical Complex	11	RM 82, RM83

2.21.1.2 Identification of fungi

The isolated selected fungus was identified morphologically as fungal colony, color, growth pattern, diffusible pigments, hyphae, conidia and sporulation.

2.21.1.3 Antibacterial activities by Fungi

All isolated fungal strains were cultured on potato dextrose agar media and incubated for 120 hours at 28 ^{o}C. Isolated pathogenic bacteria (selected strain) were transferred in 50 ml broth media, incubated in shaking incubator at 37 ^{o}C for 24 to 48 hrs. After incubation period broth culture (5 ml) was taken in culture tubes, turbidity of test bacteria was adjusted with Mc Farland solution by adding normal saline. Uniform lawns were prepared on solid nutrient agar media from diluted cultures using cotton swabs.

Cork borer of 8 mm diameter was used for taking agar medium from incubated fungal culture on PDA media in the form of circular pustules and carefully placed on prepared lawn of test bacterial strains at equal distance. Petri plates were placed in the incubator for 24 to 48 hours at 37 ^{o}C. Zone of inhibition around the pustule were recorded for different pathogenic strains.

2.22 Antibacterial activity by actinomycetes

Screening of isolated actinomycetes was carried out through cross agar streaking to assess its antimicrobial activity. All isolated actinomycetes were selected for primary screening. Actinomycetes were streaked in single line at the center of nutrient agar medium and incubated for 2 to 3 weeks at 28 ^{o}C and then perpendicularly streaked all isolated pathogenic bacteria, incubated for 24-48 hours at 37 ^{o}C.

2.22.1 Secondary screening through agar well diffusion assay

All positive actinomycetes at primary screening were subjected for secondary screening. Positive actinomycetes at primary screening were then inoculated in the starch casein nitrate broth media incubated in the orbital shaker at 28 ^{o}C for 2-3 weeks. Fermented samples were taken after every 24 hours, centrifuged at 10000 rpm for 10 mints at 4 ^{o}C and crude extract was tested through agar well diffusion method against indicator bacterial lawn prepared on Muller Hinton agar media. Selected active actinomycetes were processed for identification and one most potent isolate was selected for optimization of different parameters for maximum production of antimicrobials.

2.23 Optimization of parameters

The process in which the most favorable conditions were idealized for best growth and production of secondary metabolites by microorganisms is called optimization of parameters. In batch fermentation, fermentation product does not only depend upon the type of microorganism in question and nature of media utilized for fermentation but also culture conditions which plays a vital role [130].

All batches were optimized in duplicates in orbital shaker incubator. 250 ml Starch casein nitrate broth media was prepared in the 500 ml flasks sterilized in autoclave at 121 ^{o}C, 15 psi pressure for 15 minutes. 10% Inoculum was prepared in the sterilized starch casein broth media in 250 ml flasks. Production media was inoculated and placed in the orbital shaker incubator at 28 ^{o}C for one week. Samples were taken at every 24 hours interval and centrifuged. The pellets were discarded and supernatant was used against indicator strains at the range of 80 µl/well. Various parameters optimized for maximum antibiotic

productions were pH, temperature, inoculum size, incubation period, D-glucose concentration and speed of orbital at revolution per minute (rpm). Different values selected for different parameters were pH, 5, 6, 7, 8 and 9 for temperature 27, 28, 29, 31, 32 and 37 ^{o}C and for inoculum size 5%, 7.5%, 10%, 12.5%, 15% and 17.5% for incubation period 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours 168 hours and 192 hours. For speed of orbital shaking incubator 100, 120, 140, 160, 180, 200 rpm were optimized. These all parameters were adjusted at mentioned values for batch fermentation; samples were taken centrifuged at 4 ^{o}C and 10000 rpm for 10 minutes. Antibacterial potentials at different parameters were checked through agar well diffusion assay against Gram positive *Staphylococcus aureus* (MRSA clinical isolate) and *Micrococcus leuteus* ATCC 10240 bacteria.

2.24 Extraction of crude antibiotics

Starch casein nitrate media was prepared in 1000 ml flasks and sterilized. Inoculum of antibiotic producing isolate was prepared in broth media and incubated for 5 days. Optimized size of inoculum was added to the sterilized production media. All optimized parameters were kept constant for maximum production of antibiotic. Total 15 L media was processed and centrifuged at 10000 rpm at 4 ^{o}C to remove cell debris in the form of pellets. Supernatant was taken and processed for extraction of crude antibiotics. 20 ml supernatant was transferred in to each of the four separating funnel, extraction was carried out by adding 20 ml each of ethyl acetate, n butanol, methanol and petroleum ether. Organic solvent layers were subjected for antimicrobial activity among which only ethyl acetate layer possessed antimicrobial activity against the indicator bacterial strains. The remaining supernatant was extracted through ethyl acetate. The ethyl acetate layer

was subjected to concentrate in vacuum at 37 ^{o}C to get dry crude antibiotic. It was extracted further to purify using solvent with increasing polarity. For this purpose petroleum ether, n-butanol and methanol were used. Antimicrobial potential of each solvent system was carried out and methanol solvent part showed maximum activity. Dried product recovered from methanolic extract was dissolved in cold methanol which yields two portions, soluble part and precipitated powder. Precipitated powder was checked against indicator bacterial strains and antibacterial activities were observed (Fig 5).

2.25 Thin layer chromatography

The chromatographic requirements for thin layer chromatography were silica gel used as a stationary phase while Methanol, Acetic acid at the ratio of 8.4 : 1.0, Methanol : Chloroform at the ratio of 9.2 : 0.8 and n-butanol : Acetic acid : Water at the ratio of 6 : 2 : 1

The plates of TLC were prepared and sample was applied. After sample application plates were developed by running the mobile phase and investigated under ultraviolet light at 254 nm. Spots developments were also observed with iodine vapors.

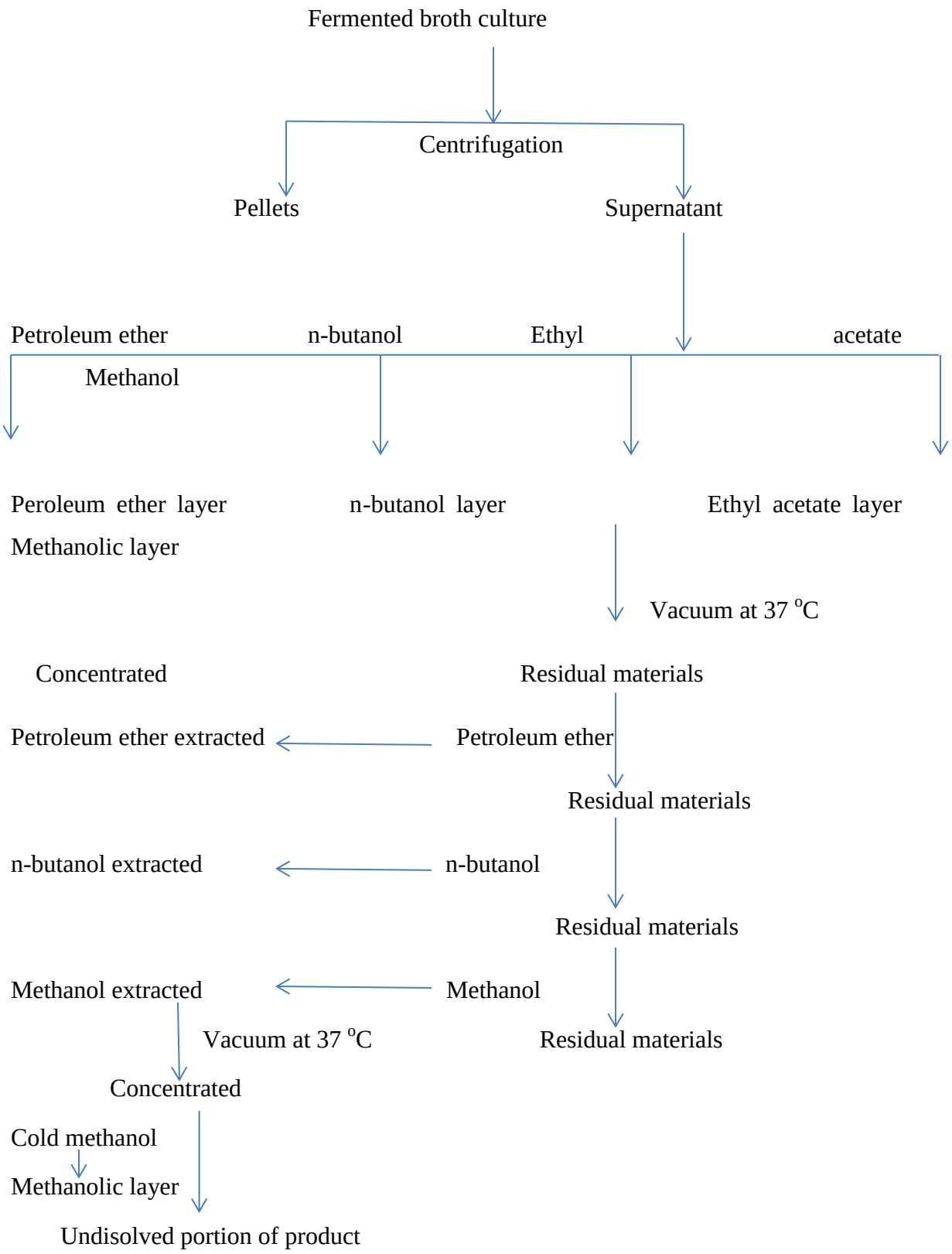

Fig 5. Solvent extraction diagram for extraction of antibiotic

2.26 Minimum inhibitory concentration (MIC) of crude antibiotics

MIC was carried out in nutrient broth medium to determine the minimum concentration of extracted antibiotic which inhibit the growth of indicator bacteria. Required materials for minimum inhibitory concentration procedure were; nutrient broth, tests tubes, pipettes, tests indicator bacterial strains, petri plates, crude antibiotic and cyclone mixer.

Fresh culture of indicator bacterial strains were serially diluted 10 times using sterile deionized water diluted bacterial strains were designated as 10^{-1} to 10^{-10}. Diluted bacteria were cultured on nutrient agar media. Cultured media was incubated for 24 hours at 37 ^{o}C. After incubation period colonies were counted and the colony forming unit were calculated in 1 ml of plated broth culture media. Stock solution of crude antibiotic was prepared by dissolving 200 mg dry powder in 20 ml DMSO to prepare 10 mg/ml stock solution. Diluted culture in nutrient broth 1.8 ml had 10^{6} CFU were added with 0.2 ml of stock solution in sterile culture tube (1). One ml was added from culture tube 1 to culture tube 2 had same concentration 1 ml cultured bacteria. Similarly other cultured tubes were serially diluted in similar manner through two fold serial dilution technique. All cultured tubes were incubated at 37 ^{0}C for 24 to 48 hrs. MICs were determined by visual observation presence or absence of turbidity due to growth of bacteria. MIC was the concentration of crude antibiotic in the unturbid cultured tubes by comparing with positive and negative controls [131].

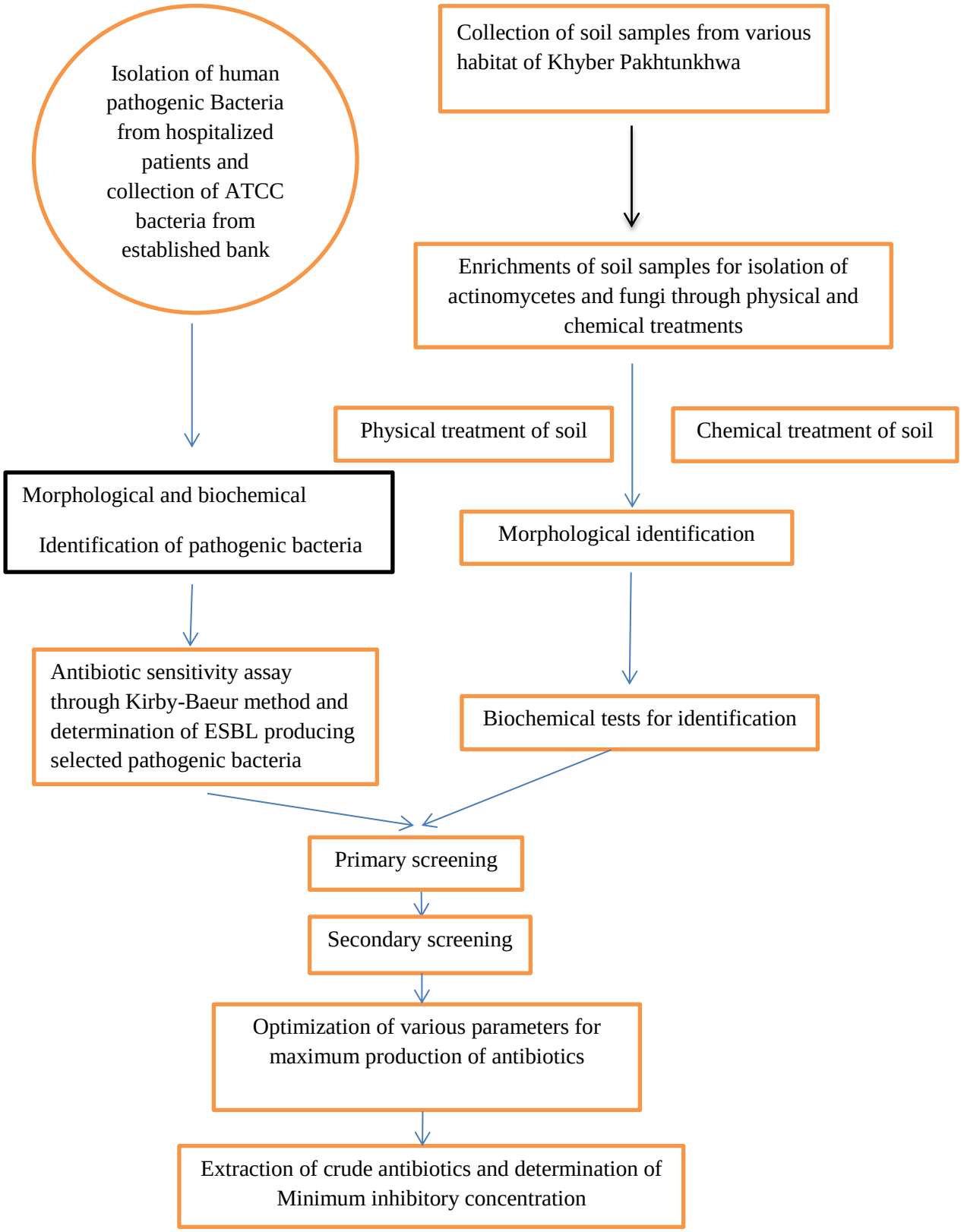

Fig 6. Schematic brief overflow chart for adopted methodology

Results and Discussion

Epidemiological study for various pathogenic bacteria revealed that major human pathogens are the *E. coli, S. aureus, S. typhi, S. paratyphi, P. aeroginosa, Enterococcus spp., Enterobactor spp., P. mirabilus, P. vulgaris, Klebsiella spp.* and *S. pneumoniae*. These pathogens were the major cause of medical complication for human in community and hospital settings because of antibiotic resistance [132].

Proper treatment in case of infectious diseases requires isolation, identification and determination of antibiotic resistance pattern for causative pathogenic bacteria. Unfortunately antibiotic resistance increased rapidly throughout the globe due to the misuse of antibiotics which limited the available options for the treatment [8-9]. Here we carried out research study at Lady Rading Hospital (LRH) Peshawar which is a tertiary care hospital, for isolation and identification of pathogenic bacteria and also performed tests for sensitivity to know resistance and susceptibility pattern towards different antibiotics tested. Furthermore antibiotic producing fungi and actinomycetes were also isolated and tested against isolated pathogens to explore new antibiotic producing microorganisms for preliminary control efforts to curb resistant pathogenic bacteria.

3.1 Bio data and samples collection from hospitalized patients

Lady Rading Hospital (LRH) Peshawar was visited during 2010 to 2011 for sample collection. Specimens were collected through proper channel. For sample collection permission was taken from admitted patients at hospital (annexure 1). Fourteen male and female wards were visited for bio data and samples collection. In this study pus, urine, blood, sputum, Cerebrospinal Fluid (CSF) and High Vaginal Swab (HVS) specimens

were collected after proper bio data information. A total of 1296 samples were collected including 618 urine samples, 510 pus samples, 50 blood samples, 60 HVS samples, 18 Cerebrospinal fluid (CSF) samples and 40 sputum samples. Age groups were in the range of 1 to 80 years admitted in various wards of hospital for different medical complications. Similar studies have been conducted earlier in which pathogenic bacteria were isolated from hospitalized patients samples including blood, urine, pus, HVS, and sputum samples collected from different age and gender groups [133]. The prevalence of pathogenic bacteria was more in females than male patients except *P. aeroginosa* in which male patients were more infected than female. Our study was consistent with previous studies conducted for isolation of pathogenic bacteria from various human samples [133-135].

3.2 Prevalence of pathogenic bacteria in hospitalized patients

Isolated pathogenic bacteria were identified on the basis of morphological features, colony color, shape, architecture, size and microscopically from cell shape and arrangements. Broadly isolated pathogenic bacteria were divided in to two groups based on Gram staining. Differential and selective media were used for differentiation among different isolates according to the recommended protocols. Biochemically isolated pathogenic bacteria were identified up to species level according to the Clinical Laboratory Standards Institute (CLSI 2006) [126] (Table 3).

Table 3. Biochemical tests for identification of pathogenic bacterial strains.

Bac Spp	U	V	O	L	Man	Glu	Suc	Ox	Cit	Mot	Ind	LDC	KIA Slop	KIA But	KIA Gas	KIA H_2S
E.C	N	N	P	P	P	P	D	N	N	P	P	P	Y	Y	N	P
P.A	N	np	np	N	np	N	Np	P	P	P	N	Np	R	R	N	N
S.T	N	N	N	N	P	P	N	N	N	P	N	P	R	Y	N	P
S.A	P	N	N	N	P	P	P	N	P	N	N	P	Y	Y	P	N
EB	N	P	P	P	P	P	D	N	P	P	N	D	Y	Y	P	N
ES	D	P	P	P	P	P	P	N	P	P	N	N	Y	Y	P	N
KN	P	P	P	P	P	P	P	N	P	N	N	P	Y	Y	P	N
PM	D	P	N	N	N	P	D	N	P	P	N	N	R	Y	P	P
PV	P	N	N	N	N	P	P	N	d	P	P	N	R	Y	d	P
SP	N	P	P	P	P	P	P	N	P	P	N	N	Y	Y	N	N

Key: Bac spp- bacterial species. U- Urease test. V- Voges-Proskauertest. O- beta-galactosidase test. L-Lactose test. Man-Manitol test. Glu-Glucose utilization test. Suc-Sucrose utilization test. Ox-Oxidase test. Cit-Citarte utilization test. Mot-Motility test. Ind-Indol test. LDC- Lysine decarboxylase test. KIA- Kligler iron agar. E.C- E. coli, P.A- P. aeroginosa, S.T- S. typhi, S.A- S.aureus, EB-Enterobacter, KN-Kleibsella pneumonia, PM- Protius mirabilus, PV- Protiun vulgarus, SP-Sterptococcus pneumonia, N=negative, P=positive, np=not performed. R=Red, Y=Yellow. d=different strains showed different results.

Total 542 pathogenic bacteria were isolated from 1296 patients at LRH Peshawar. Most prevalent pathogenic bacteria isolated from human samples were *E. coli* (43.91% n=238) followed by *S. aureus* (29.88% n=162), *P. aeroginosa* (15.68% n=85), *S. typhi* (2.95% n=16), *Enterococcus spp* (2.21% n=12), *P. mirabilus* (1.29% n=7), *Klebessiella spp.* (1.47% n=8), *P. vulgarus* (1.10% n=6), *S. pneumoniae* (1.10% n=6) and *Enterobacter spp.* (0.36% n=2), (Fig 7) and (Table 4).

Our study showed similarity with previous studies conducted in Pakistan in which *E. coli* was the prominent species isolated from clinical samples followed by *Enterococcus spp, Pseodomonas spp. Klebsiella spp., Enterobacter spp. Proteus spp.* and *Morgenella species* [136]. Our study results are also in consistent with the study conducted in Nigeria Yola hospital in which the isolated pathogenic bacteria were; *E. coli* followed by *K. pneumonia, P. mirabilis, S. faecalis, S. aureus, P. vulgaris, stuartii, S. epidermidis, A. faecalis, S. saprophyticus, P. aeroginosa, S. marsescens* and *C. fruendii* [137].

Table 4. Gender wise percentage (%) occurrence of pathogenic bacteria in hospitalized patients

Bacteria	**Total (N)**	**%**	**Male (N)**	**%**	**Female(N)**	**%**
E. coli	238	43.91	112	47.03	126	52.93
S. aureus	161	29.88	80	49.68	81	50.31
Enterobacter spp.	2	0.36	1	50	1	50
P. aeroginosa	85	15.68	57	67.05	27	31.76
S. typhi	16	2.95	7	43.75	9	56.25
Enterococcus spp.	12	2.21	4	33.33	8	66.66
P. mirabilus	7	1.29	2	28.57	5	71.42
P. vulgarus	6	1.10	3	50	3	50
S. pneumonia	6	1.10	2	28.57	5	71.42
Kliebessella spp.	8	1.47	3	37.5	5	62.5
Total	542	100	271	50.18	270	49.81

Key: N, number of isolated pathogenic bacteria.

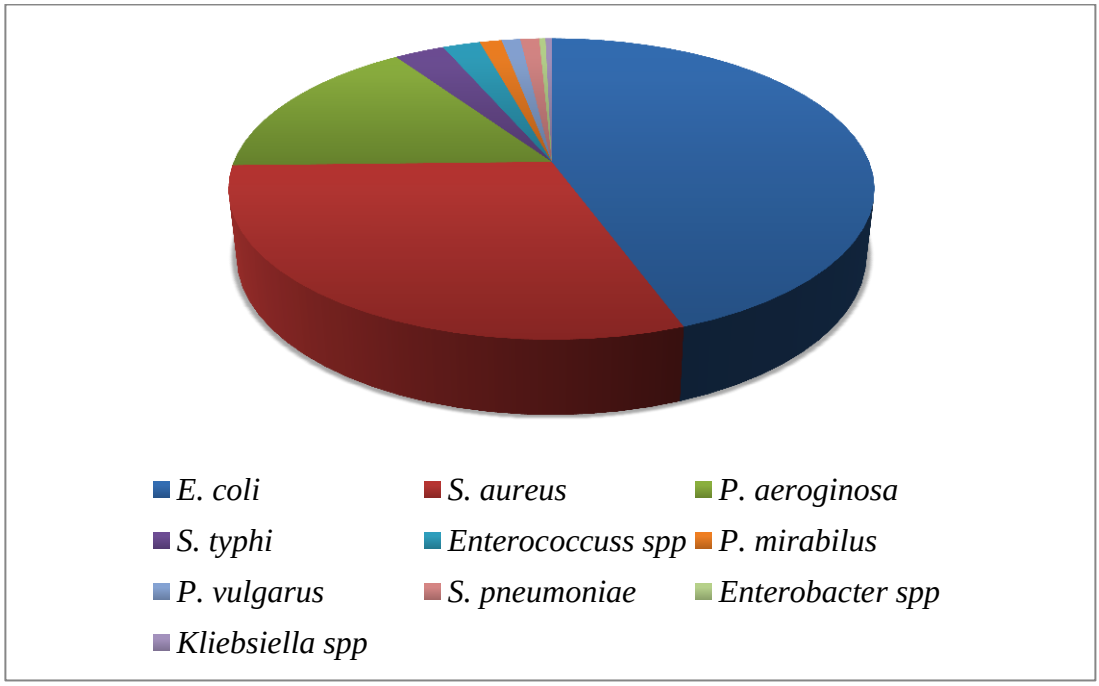

Fig 7. Prevalence of isolated pathogenic bacteria in hospitalized patients.

3.3 Prevalence and resistance pattern of *E. coli*

In this study 238 *E. coli* strains were isolated from pus, blood, urine, CSF and HVS samples collected from different wards of LRH Peshawar. Out of 238 positive samples, females (52.93%) were more infected with *E. coli* than male (47.03%) (Figure 8). Majority of *E. coli* strains were isolated from urine (44.53 %) followed by pus, HVS, blood and cerebrospinal fluid (CSF) (Table 5). Our study is in consistent with the previous study conducted in Lahore Pakistan for isolation and gender wise prevalence of *E. coli* [138].

Table 5. Number of *E. coli* isolated from hospitalized patients samples (n=238).

Urine	Pus	HVS	Blood	CSF	Total
107	76	40	10	5	238

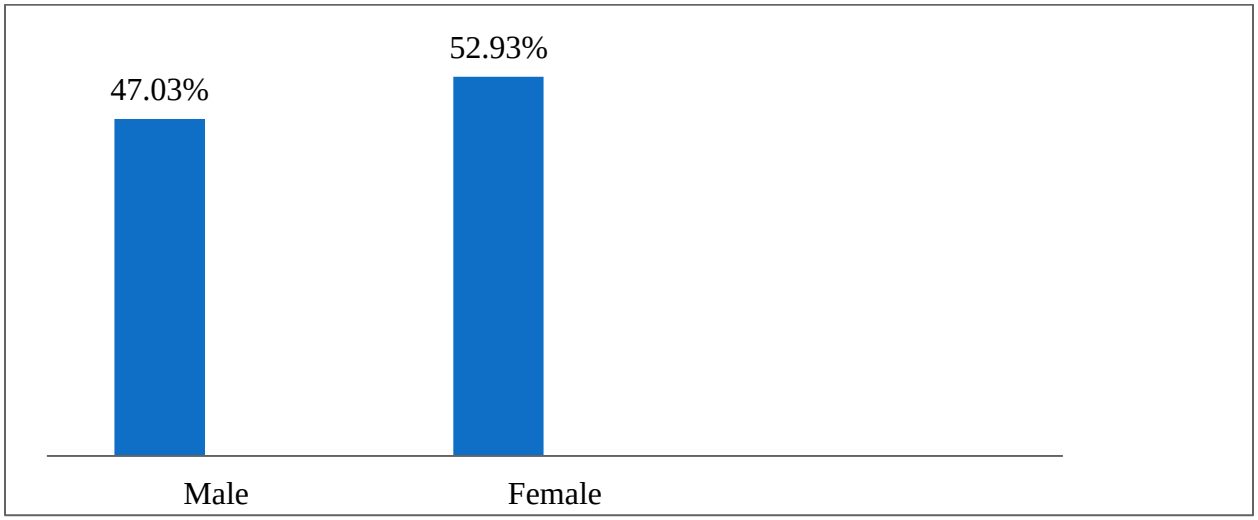

Fig 8. Gender wise prevalence of *E .coli* isolated from hospitalized patients (Percent bar).

E. coli was found the most prominent pathogenic bacteria in all isolated pathogens in this study followed by *S. aureus, P. aeroginosa, S. typhi, Enterococcus spp., Streptococcus spp., P. vulgaris, Enterobacter spp.* and *P. mirabilus.* Antibiotic resistance pattern of *E. coli* revealed in our study that a number of antibiotics were in-effective while only a few of them were comparatively more effective against the isolated *E. coli* strains. The percent resistance of *E. coli* to various antibiotics tested included those with very low efficacy Cephradine (96.63%), Cefuroxime (85.29%), Cephtriaxone (78.15%), Ceftazidime (75.21%), Coamoxiclave (72.68%), Cefotaxime (70.58%), Nalidixic acid (68.48%), Levofloxacin (62.18%), Ciprofloxacin (60.50%), Sparfloxacin (58.40%), Ofloxacin (57.98%), moderate efficacy Pipemedic acid (38.23%), Moxifloxacin (33.61%), Amikacin (22.60%), and comparatively higher efficacy Piperacillin/Tazobactam (8.40 %), Cefoperazone / Sulbactam (7.50%), Meropenem (3.70%), and Imipenem (2.94%). Most potent antibiotics were Cefoperazone / Sulbactam (7.50%, Meropenem 3.70%, and Imipenem 2.94% while less potent antibiotics were Cephradine 96.63%,) and Cefuroxime (85.29%), (Fig 9).

Imipenem was the most potent antibiotics tested against *E. coli* in Iran [139-141]. Our results also showed similarity in some antibiotics used by [138] which indicated that *E. coli* showed resistance to different antibiotics including Ampicillin (92%), Cotrimoxazole (86%), Ciprofloaxacin (80%), Gentamicin (62%), Nitrofuratoin (47%) and Amikacin (4%). Another study conducted by [59] showed that most effective antibiotic was Imipenem which was 100% effective tested against *E. coli.* Total 121 *E. coli* were isolated from patient's samples and investigated for antibiotic sensitivity. All isolates were sensitive to Imipenem, (60%) resistance was observed against Cefoxitin, while

resistance to Cefotaxime, Ciprofloxacin and Cefepime were (89%), (87.6%) and (87%) respectively.

Research findings from developed and developing countries revealed that irrational use of antibiotics is the main reason of antibiotic resistance contributing in treatment failure. For instance Penicillin was the drug of choice when discovered, soon resistance developed to this antibiotic, another antibiotic groups like Floroquinolon and Cephalosporin replaced the previous one but unfortunately resistance to these drugs were also reported [142, 143]. Research study revealed from various countries in which *E. coli* showed resistance to betalactam Cotrimoxazole and Ampicillin [144, 145]. In developing countries pathogenic bacteria gradually arises resistance towards Floroquinolon group of antibiotics creating some serious concern among clinical practitioners and health authorities because infected patients in these poor countries cannot afford lab tests and regular visits to hospitals, due to which they depends on the empirical therapies which is not effective practice of treatment. Studies also revealed that even in the developed countries like USA, antibiotics are prescribed irrationally and unwisely and is the cause of emerging resistance [146, 147]. Present research findings are in consistent with the reports from Turkey, Senegal, Australia India Slovenia and Brazil [148-150]. High rate of resistance might be due to the irrational use of antibiotics during chemotherapies and because of horizontal transfer of resistant genes from resistant organisms to sensitive one via plasmids, transposons, bacteriophages and integrons, plasmids or transposons can carry several resistant genes simultaneously. In this way pathogenic microorganisms became resistant to several antibiotics and so called multidrug resistant bacteria are developed. Some time when a microorganism develops resistance to one drug, at the

same time, also develops resistance to another antibiotic. To solve this adverse situation in developing countries, clinicians should prescribe correctly targeted antibiotics with appropriate required doses, and health authorities should conduct regular supervision on the antibiotic prescriptions and utilization [151]. In the present study, (84%) isolated *E. coli* were resistant to three or more than three antibiotics and especially against Cephradine, Cefuroxime and Ceftriaxone. The Multi drugs resistance percentage changes with time to time and geographical location; in 2000 (7.1%) MDR *E. coli* were found in USA while it was (42 %) in Slovenia studied in 2006. In Pakistan, study conducted in Lahore revealed that 65.5% *E. coli* were found resistant to 8 or more than 8 antibiotic tested. Antimicrobial drugs used in this study belonged to three different classes of antibiotics [152]. Another study in Iran showed that (73%) *E. coli* were resistant to three or more than three antibiotics most notably to Ampicillin, Tetracycline and Cotrimaxazol [153].

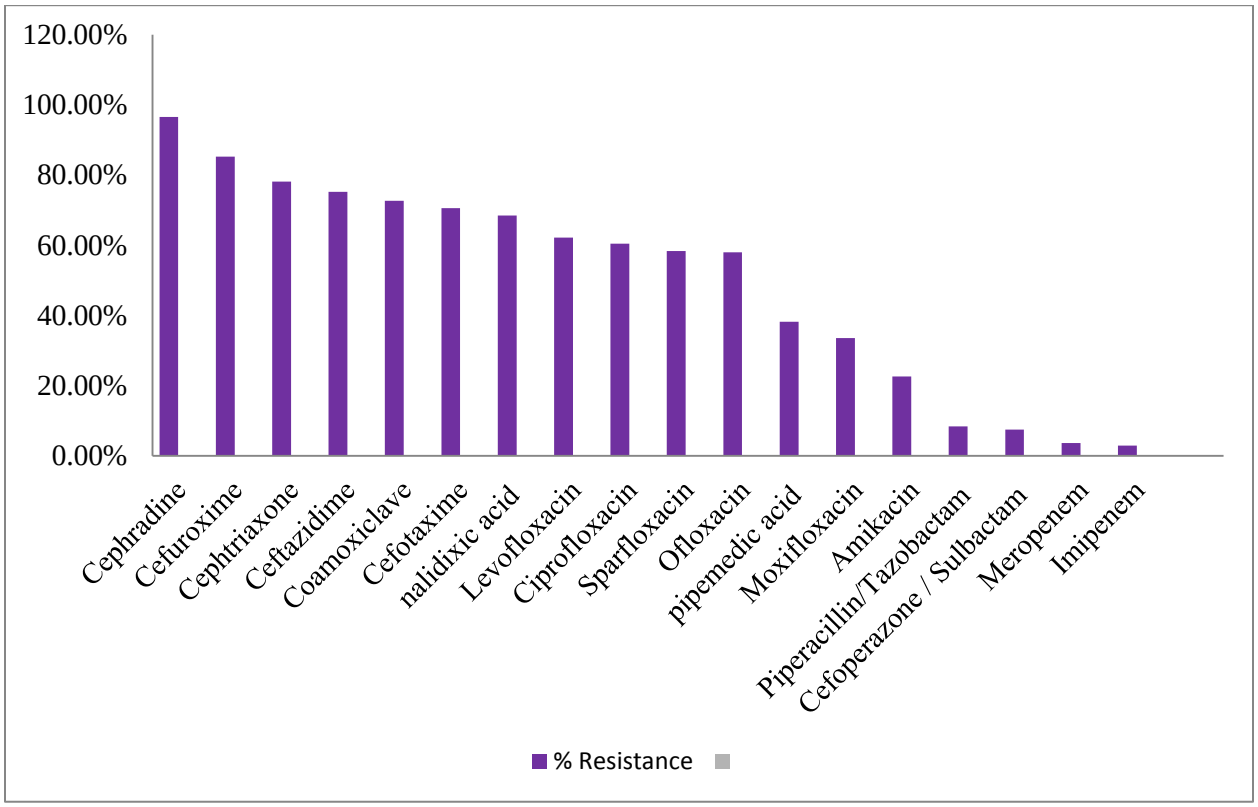

Fig 9. Percentage bar for resistance pattern of various antibiotics tested against *E. coli*.

Antibiotic resistance study conducted in different countries revealed that *E. coli* resistance pattern in India to antibiotics like Cotrimoxazole, Tetracycline, Chloramphenicol, Nalidixic acid, Gentamicin, Ceftazidime, Ciprofloxacin, Amikacin and Imipenem tested were (48.3%), (39.7%), (10.3%), (55.2%), (10.2%), (3.4%), (22.4%), (0.0%) and (0.0%) respectively. In USA, antibiotic resistance studies showed that *E. coli* was resistant to Cotrimoxazole (40.4%), Nalidixic acid (29.3%), Gentamicin (8.4%), Norfloaxacin (11%), while the strains were susceptible to Amikacin (0.0%) and Imipenem (0.0%). Almost similar results were obtained in Australia for various antibiotics such as Cotrimoxazole (36%), Tetracycline (30%0, Norfloxacin (5.8%), Amikacin (2.5%), Imepenem (0.0%). Antibiotic resistance study for *E. coli* infection conducted in Iran indicated that resistance had developed even in the case of antibiotics reported previously to be highly efficient against *E. coli*. The overall pattern of resistance was as follows; Cotrimoxazole (81.1%), Tetracycline (75.5%), Chloramphenicol (37.7%), Nalidixic acid (26.6%), Gentamicin (16.6%), Ceftazidime (11.1%), Ciprofloaxacin (8.8%), Norfloaxacin (8.8%), Amikacin (3.3%), Imipenem (1.8%) [154-156]. Recent study conducted in Pakistan Lahore revealed antibiotic resistance pattern against *E. coli* for Imipenem (0.0%), Meropenem (0.7%), Piperacilline/Tazobactam (3.2%), Amikacin (10.3%), Cefuperazone/Sulbactum (13.2%), Ceftazidime (22.8%), Gentamicin (26.8%) and Aztreoname (32.9%) while the rest of tested antibiotics showed 51 to 96% resistance [152]. Surprisingly our study revealed 2.94% resistance when Imipenem antibiotic was tested against isolated *E. coli*, which indicates that resistance to Imipenem is building up in KPK.

Higher resistance pattern to various antibiotics tested in our study in KPK might be due to improper prescription of antibiotics, lack of government policy regarding the prescription of antibiotics, self-medication and lack of awareness about the risk of using various antibiotics. To control resistance, it is recommended to implement strict regulations and regular supervisions of health providing facilities to prescribe proper targeted antibiotic in sufficient doses. It is also necessary to prohibit antibiotic sale without prescription. Isolation of patients suspected for the causative resistant pathogenic bacteria is also a better way to control the spread of resistant genes to the sensitive bacteria. Research is also needed on regular basis to accumulate sufficient data to know the exact percentage of resistance for various infectious bacteria to adopt strategies accordingly in order to stop resistance or at least to slow down the spread of resistance pattern. To reduce the rate of resistance it is worthwhile needed to search new sources of antibiotic producing microorganisms or production of new semisynthetic drugs or phage therapies.

3.3.1 Isolation and characterization of ESBL producing *E. coli*

Extended spectrum beta lactamase (ESBL) producing pathogenic bacteria developed a great problem to therapeutics. Frequency rate of ESBL producing *E. coli* has increased rapidly, during the past few years. Total 15 ESBL producing *E. coli* were characterized for antibiogram using various antibiotic discs. ESBL producing *E. coli* were resistant to Gentamicin (100%), Cephradin (100%), Meropenem (0%), Ciprofloaxacin (66.66%), Cephtriaxone (86.66%), Coamoxiclave (100%), Pipemedic acid (100%), Nalidixic acid (100%), Cefotaxime (93.33%), Ceftazidime (93.33%), Cefurixine (100%), Ofloaxacin (86.66%), Amikacin (60%), Sparfloaxacin (73.33%), Levofloaxacin (60%), Imipenem

(0%), Norfloaxacin (86.66%). Our results are in consistent with research study on isolation of ESBL producing *E. coli* from hospitalized patients in Shahid Bohonar hospital situated in Iranian capital city Tehran [58]. *E. coli* capable of ESBL production had emerged during the last few decades which became a wide spread pathogens especially in hospital acquired infections throughout the world. It is clear from recent research findings that infection caused by ESBL producing bacteria had higher fatality rate than those bacteria which did not produced ESBL [49]. ESBL producing *E. coli* frequency vary from one region of the world to another, and also quickly changing over different periods [157]. Our study showed that ESBL producing *E. coli* was (100%) sensitive to Imipenem and Meropenem. Isolated ESBL producing *E. coli* were (100%) resistant to Cefuroxime, Nalidixic acid, Pipemedic acid, Cephradin and Gentamicin. This higher resistant might be due to ESBL producing gene located on plasmid which can easily transfer to sensitive bacterial population and progeny and can take genetic materials coding resistance for various antibiotic groups. To control the complication regarding the ESBL producing bacteria physicians must prescribe antibiotics with care particularly to avoid uncontrolled prescription of oral Cephalosporin (Cefurixime) which was (100%) resistant in our findings (Table 6).

Table 6. Percentage resistance and sensitivity of ESBL producing *E. coli* isolates

Antibiotics used	Sensitivity %	Resistivity %
Gentamicin	0	100
Cephradin	0	100
meropenem	100	0
Ciprofloaxacin	33.34	66.66
Cephtriaxone	13.34	86.66
Coamoxiclave	0	100
Pipemedic acid	0	100
Nalidixic acid	0	100
Cefotaxime	6.67	93.33
Ceftazidime	6.67	93.33
Cefurixine	0	100
Ofloaxacin	13.34	86.66
Amikacin	40	60
Sparfloaxacin	26.67	73.33
Levofloaxacin	40	60
Imipenem	100	0
Norfloaxacin	13.34	86.66

3.4 *Staphylococcus aureus* antibiotic resistance pattern

S. aureus caused hospital and community acquired infections throughout the globe and have high mortality rate in spite the prescription of potent antibiotics. In the present study *S. aureus* were investigated for antibiogram. *S. aureus* isolated from pus, HVS and blood samples from hospitalized patients, resistance to antibiotics were found significantly high. In our findings, higher number of *S. aureus* strains were isolated from pus samples (87.57%) followed by HVS (9.31%) and blood (3.10%), (Table 7). Total 161 pathogenic *S. aureus* were isolated which showed higher resistance percentage to large numbers of antibiotics tested. Methicillin resistant *S. aureus* isolates were 86 out of 161 (53.41%) isolated *S. aureus*. The percent resistance of *S. aureus* to various antibiotics tested included those with very low efficacy, Cotrimixazol (94.19%), Klathromycin (71.42%), Levofloaxacin (66.45%), Sparfloaxacin (65.56%), Cifrofloaxacin (65.21%), Cefotaxime (59.68%), Cephradin (57.76%), Cephtriaxone (54.65%), Ofloaxacin (54.65%), Meropenem (53.70%), Imepenem (53.70%), Oxacillin (52.44%), Coamoxiclave (52.46%), and antibiotics with moderate efficacy were Doxicyclin (39.86%), Chloramphenicol (21.11%) Gentamicin (16.66%) and Fusidic acid (8.60%), most potent antibiotics revealed resistance percentage were Linezolid (1.20%) Vancomycin (0%) Taecoplanin (0%) (Table 8), (Figure 11). These outcomes showed that Tacoeplanin, Vancomycin and Linezolid were the most effective antibiotics. While Oxacillin, Meropenem, Imipenem, Klathromycin, Cephradin, Levofloxacin, Cotrimixazol, Cephtriaxon, Sparfloaxacin were the least effective antibiotics against isolated *S. aureus.* Our study has similarity with research findings conducted in Iran Pakistan and Nigeria for at least the most potent antibiotics [27, 158, 159]. In the present

study, 52.44 % *S. aureus* were resistant to Oxacillin which is the worse indication of hospital acquired MRSA prevalence in KPK. It indicates that MRSA have emerged to endemic proportion in our hospitals. Prevalence rate of MRSA changes with time and in different countries even in the same countries with different localities. A study conducted in neighboring country Iran in which MRSA prevalence was 33% [160]. A study carried out from 1996 to 1999 in US hospitals that Oxacillin resistant *S. aureus* increased up to 2.4% during study period [161]. Prevalence of MRSA in Austria 21.6%, Spain 30.3%, Belgium 25.1% and in French hospitalized patients 33.6% was reported [162]. A study conducted in Pakistan Institute of Medical Sciences (PIMS) Islamabad reported that 61.29 % *S. aureus* were resistant to Oxacillin [163].

Table 7. Number of isolated *S. aureus* from different collected samples from hospitalized patients.

Pus	HVS	Blood	Total
141	15	5	161

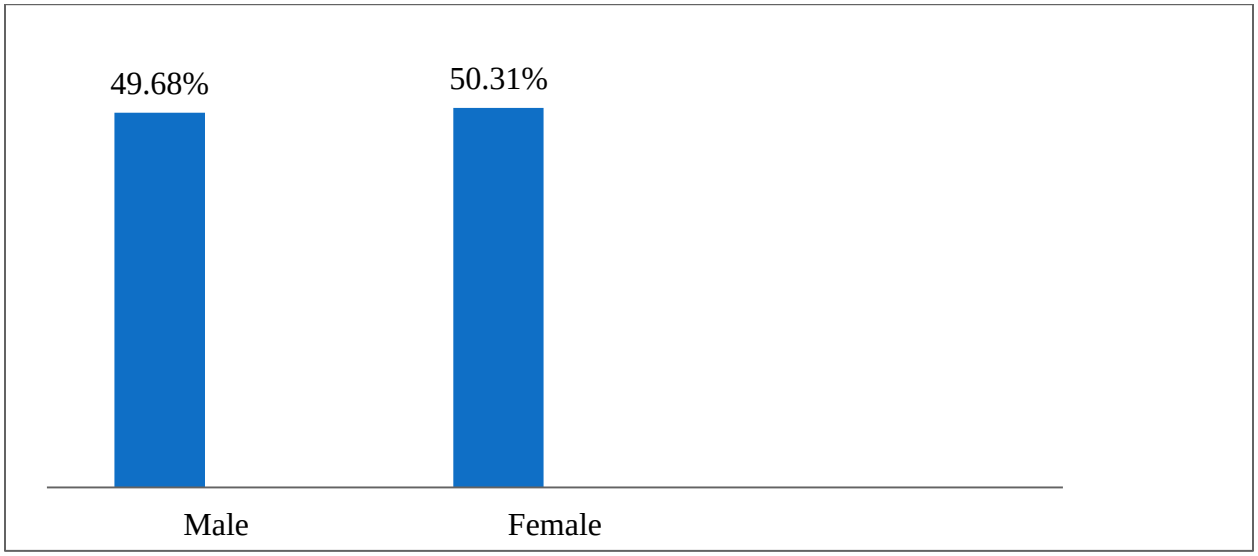

Fig 10. Male, female percent bar for *S. aureus* infection in hospitalized patients

Table 8. Antibiotic sensitivity and resistance pattern of *S. aureus* against different antibiotics.

Antibiotic used	**Resistant**	%	**Susceptible**	**%**
Coamoxiclave	85	52.46	76	47.24
Gentamicin	27	16.66	134	83.22
Meropenem	87	53.70	74	46.30
Imipenem	87	53.70	74	46.30
Cephtriaxone	88	54.65	73	45.35
Cifroflaxacin	105	65.21	56	34.79
Ofloaxacin	88	54.65	73	45.35
Oxacillin	75	52.44	86	47.56
Doxicyclin	61	39.86	100	60.14
Klathromycin	115	71.42	46	28.57
Cefotaxime	77	59.68	84	40.32
Cephradine	93	57.76	68	42.24
Levofloaxacin	107	66.45	54	33.55
Sparfloaxacin	99	65.56	62	34.44
Linezolid	2	1.20	159	98.80
Vancomycin	0	0	161	100
Taecoplanin	0	0	161	100
Fusidic acid	14	8.60	147	91.40
Cotrimixazol	146	94.19	15	5.81
Chloramphenicol	34	21.11	127	78.89

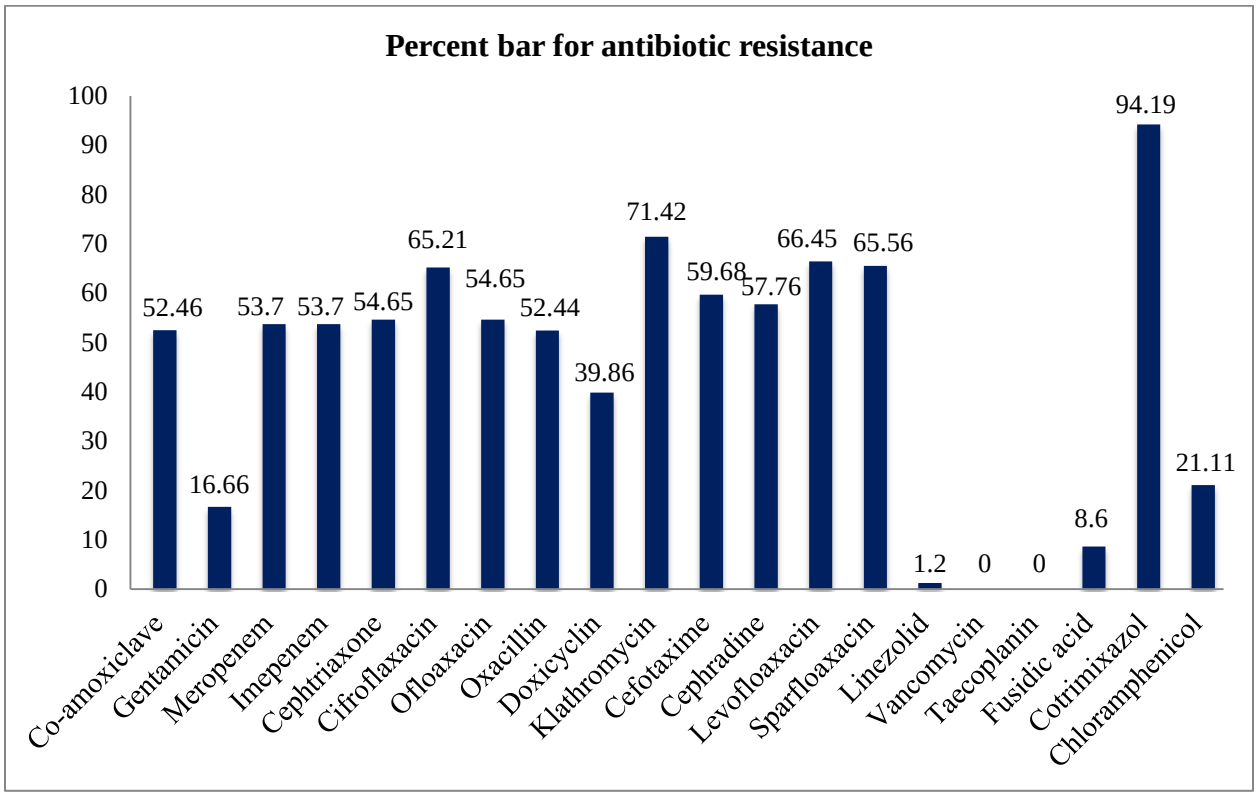

Fig 11. Resistance percentage to different antibiotics tested against *S. aureus*.

3.5 *Pseudomonas aeroginosa* Resistance pattern towards different antibiotics

In the present research study conducted in LRH Peshawar total 85 *P. aeroginosa* were isolated from patients in various wards. *P. aeroginosa* were isolated from Pus, Urine, blood, ear swab and HVS samples. Majority of pathogens were isolated from pus samples followed by blood, urine, ear swab and HVS samples (Fig 13). Out of 85 isolated pathogens 57 were male patients (67.05%) and 27 were female patients (32.94%), (Fig 12). Our study is in consistent with a study conducted in Dera Ismail Khan Combined Military Hospital (CMH) Pakistan in which similar outcomes were observed. Prominent numbers of *P. aeroginosa* were isolated from pus samples followed by other collected specimens [164]. Another study showed similar results in gender wise prevalence for isolated *P. aeroginosa* in which Male were 70.80% infected and females were 29.10% infected [165].

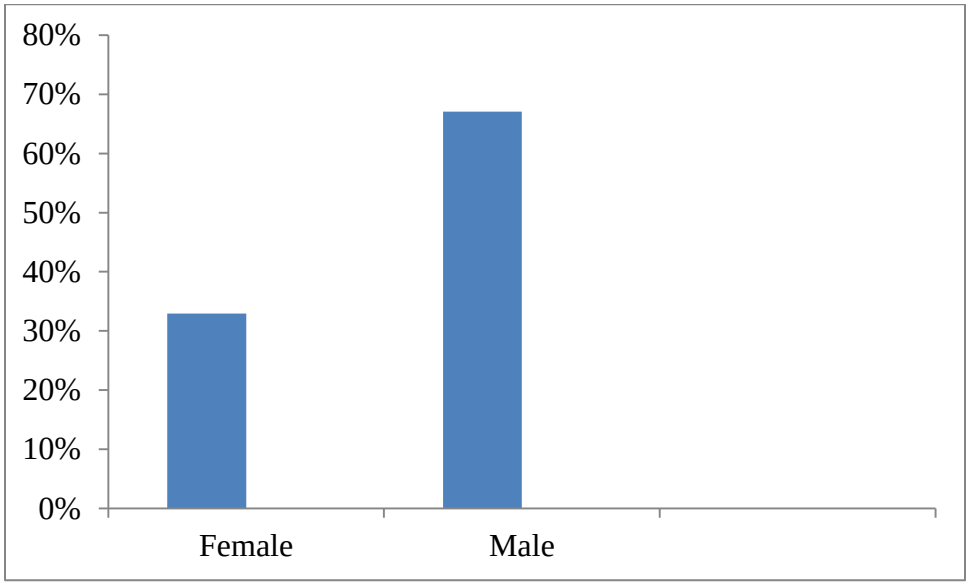

Fig 12. Percent bar for gender wise prevalence in isolated Pathogenic *P. aeroginosa* in hospitalized patients.

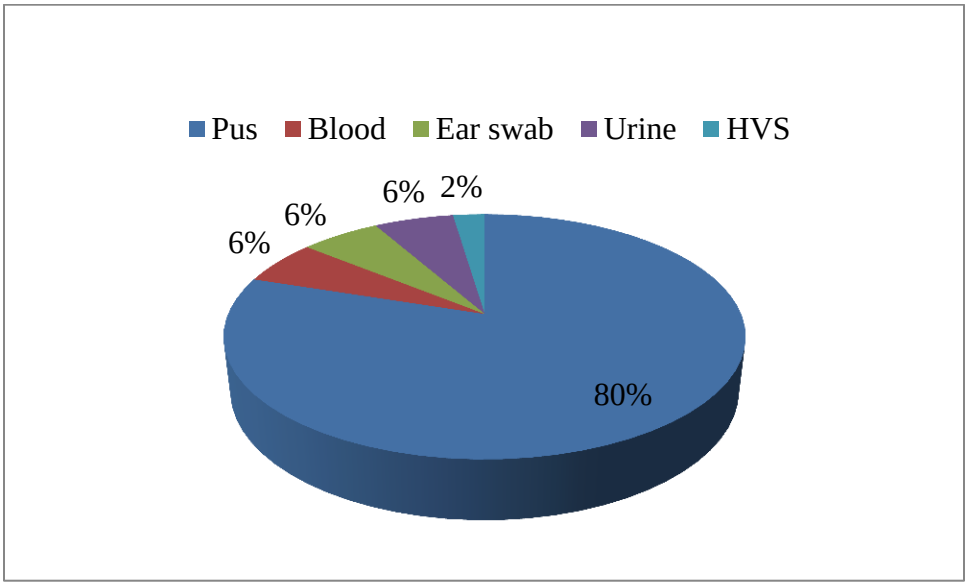

Fig 13. Percent pie chart showed isolated *P. aeroginosa* from different samples.

P. aeroginosa is an opportunistic pathogen which caused infection mainly in immuno compromised patients especially hospitalized for longer period of time and using broad spectrum antibiotic therapy [166, 167]. *P. aeroginosa* caused infection in skin, sepsis and largely respiratory tract infections in hospitalized patients [166, 167]. Mechanisms of antibiotic resistance in *P. aeroginosa* are due to the enzyme betalactamase production and the functioning of Multiple efflux pump mainly "MexAB.OprM" system responsible for efflux [168]. Now a day MRSA and MDR *P. aeroginosa* received progressive attention among clinicians [164].

In the present study 14 antibiotics were tested against *P. aeroginosa.* Resistance towards these antibiotics were shown in the (Figure 14) and (Table 9). Our results revealed that most potent antibiotics Imipenem showed (8.23 %) resistance pattern, Piperacillin/Tazobactam revealed (10.58%), resistance, Meropenem was (7.05%) and Cefoperazone/Sulbactam revealed (17.64%) resistance pattern. Our results correlates with another study conducted in Pakistan [164] in which Tazobactam/Piperacillin were the most effective antimicrobials agents in case of *P. aeroginosa* infections. In another study findings revealed that Imipenem and Amikacin were the most effective antibiotics for *P. aeroginosa* infection. In our study Imipenem was the most effective antibiotic than Amikacin. This might be due to the over prescription of this antibiotic in case of *P. aeroginosa* infection.

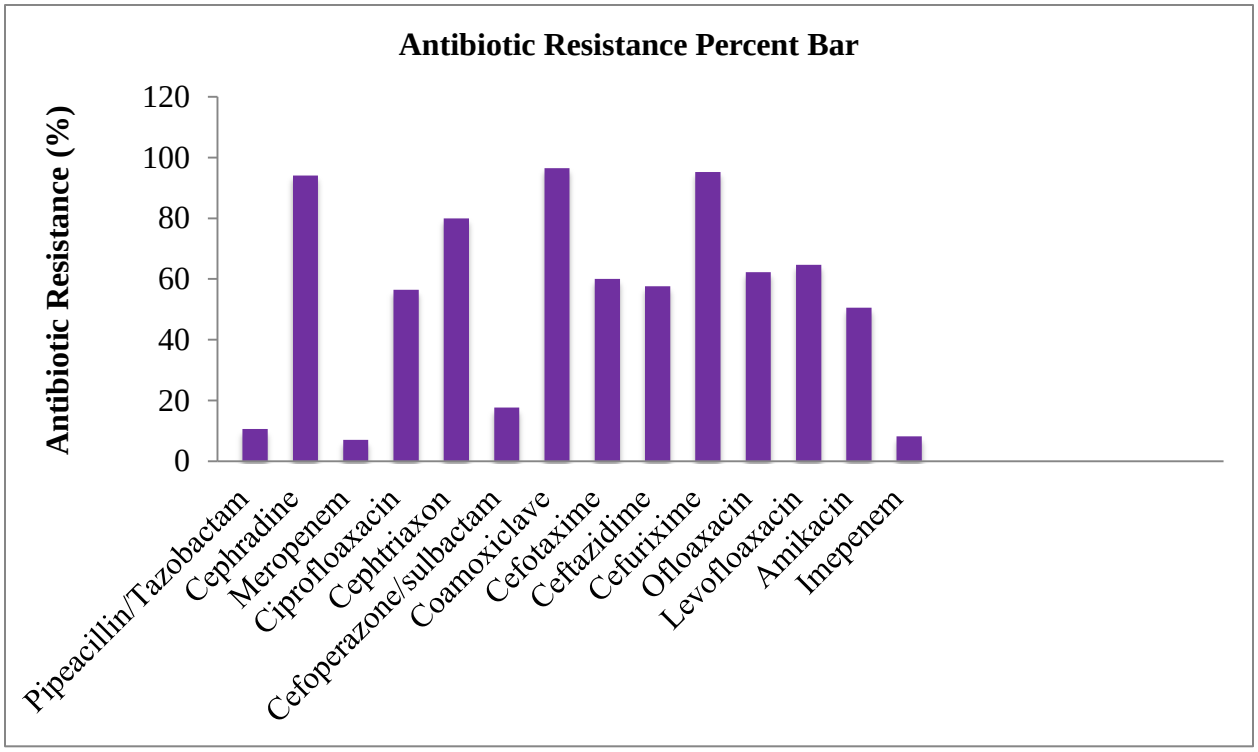

Fig 14. Antibiotic resistance percentage for different antibiotics tested against *P. aeroginosa*.

Table 9. Antibiotic resistance and susceptibility pattern of *P. aeroginosa*

Antibiotics used	**Resistant**	%	**Susceptible**	**%**
Meropenem	6	7.05	79	92.95
Imipenem	7	8.23	78	91.77
Cephtriaxone	68	80	17	20
Cifrofloaxacin	48	56.47	37	43.53
Ofloaxacin	53	62.35	32	37.65
Cefotaxime	51	60	34	40
Cephradine	80	94.11	5	5.89
Levofloaxacin	55	64.70	30	35.30
Coamoxiclave	25	94.47	60	5.53
Amikacin	43	50.58	42	49.42
Cefurixime	81	95.29	4	4.71
Ceftazidime	49	57.64	36	42.36
Cefoperazone/Sulbactam	15	17.64	70	82.36
Piperacillin/Tazobactam	9	10.58	76	89.42

It is clear from our findings that *P. aeroginosa* was resistant to many antibiotics highest resistance pattern was noted in case of Cefurixine (95.29%), coamoxiclave (96.47%), Cephradin (94.11%) and Cephtriaxone (80%). The most effective antibiotics were Imipenem, Cephoferazon/Sulbactam, Meropenem and Piperacillin/Tazobactam showed (8.23%), (17.64), (7.05%) and (10.58%) resistance pattern respectively. Our results were comparable with a previous study in which Imipenem was the most effective antibiotic utilized against *P. aeroginosa* [169]. In another study carried out on 81 *P. aeroginosa* isolated from hospitalized patients in which Amikacin was the most effective antibiotic (8% resistance pattern) next potent antibiotic was meropenem with 78% susceptibility [170]. Another study carried out by Macedo and Santos that Amikacin and Imipenem was the most potent antibiotic for the *P. aeroginosa* infected patients [171].

3.6 Isolation and identification of fungi for antimicrobial activities against MDR bacteria

Total 9 fungal strains were isolated from 33 soil samples collected from various hospitals of Peshawar. Only one strain was screened out in the primary screening active against Multi drugs resistant (MDR) isolated pathogenic bacteria. Rest of the isolated fungal strains showed no activities against isolated pathogenic strains. The bioactive compounds producing fungi against pathogenic bacteria was identified using different monographs and manuals which based upon the morphological and colony characteristics, like growth pattern, fluorescence of growth under ultra violet light, color and exudates produced during growth and structure of spores developed, like conidia, sporangia, pycnidia, accervuli, sporodochia and ascocarps [172-173]. On the basis of above characteristics the fungal strain was primarily identified as *Aspergillus ochraceus*.

3.6.1 Antimicrobial spectra of isolated fungi

Isolated potent fungal strain was tested against 45 isolated human pathogenic bacterial strains in the primary screening active against both Gram positive and Gram negative bacteria. Zone of inhibition around 8 mm diameter agar pustule containing diffused bioactive metabolites of potent fungal strain placed on the prepared lawn of Gram positive pathogenic bacteria was 15 to 20 mm zone of inhibition showed significant results. Antimicrobial activities were also observed against Gram negative isolated bacteria from hospitalized patients which showed 10 to 15 mm zone of inhibition except *E. coli* which revealed less than 10 mm zone of inhibition (Table 10). The indicator bacterial strains were isolated from hospitalized human patients already identified on the basis of morphological and biochemical characteristics were *S. aureus, P. aeruginosa, S. typhi, Streptococcus spp., Klebsiella spp.* and *E. coli.* These isolated indicator bacterial strains were resistant to two or more than two antibiotics which were categorized as multi drugs resistant (MDR). Isolated *A. ochraceus* revealed bioactivity against all 45 tested bacterial strains (Table 10).

Table 10. Antibiotic sensitivity assay and antimicrobial activity of isolated *A. ochraceus*, against isolated pathogenic bacteria

Indicator Bacteria	Antibiotic discs used										Fungi
	CE	CEP	LEV	SPA	LINE	VAN	TEIC	FUS	COT	CHL	RM82
Klebsiella spp.	Re	Re	Re	Re	Se	Se	Se	Se	Re	Se	+
Klebsiella spp.	Re	Re	Se	Se	Se	Se	Se	Se	Re	Se	+
Klebsiella spp.	Re	Se	Se	Re	Se	Se	Se	Se	Se	Se	+
Klebsiella spp.	Re	Se	Se	Se	Se	Se	Se	Se	Re	Se	+
Klebsiella spp.	Se	Re	Se	Se	Se	Se	Se	Se	Se	Se	+
Klebsiella spp.	Se	Re	Se	Se	Se	Se	Se	Se	Re	Se	+
Streptococcus spp.	Re	Se	Se	Se	Se	Se	Se	Se	Re	Se	+
Streptococcus spp.	Se	Se	Se	Se	Se	Se	Se	Se	Re	Se	++
Streptococcus spp.	Re	Se	Se	Se	Re	Se	Se	Se	Se	Se	+
Streptococcus spp.	Se	Re	Se	Se	Se	Se	Se	Se	Re	Se	+
P. aeruginosa	Re	Re	Re	Re	Se	Se	Se	Se	Se	Se	+
P. aeruginosa	Re	Re	Re	Se	Se	Se	Se	Se	Se	Se	+
P. aeruginosa	Se	Re	I	Re	Se	Se	Se	Se	Se	Se	+
P. aeruginosa	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	+
P. aeruginosa	Se	Re	Re	Re	Se	Se	Se	Se	Se	Se	+
P. aeruginosa	Re	Re	I	Se	Se	Se	Se	Re	Se	Se	+
S. aureus	Se	Se	Se	Se	Se	Se	Se	Re	Re	Se	++
S. aureus	Re	Se	Se	Re	Se	Se	Se	Se	Re	Se	++
S. aureus	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	++
S. aureus	Re	Se	Re	Re	Re	Se	Re	Se	Se	Se	+

S. aureus	Re	Re	Re	Re	Se	Se	Se	Re	Re	Re	+
S. aureus	Re	Se	Re	Re	Se	Se	Se	Re	Re	Re	++
S. aureus	Re	Re	Re	Re	Se	Se	Se	Se	Re	Re	+
S. aureus	Se	Re	Se	Se	Se	Se	Se	Se	Re	Se	++
S. aureus	Re	Se	Se	Re	Re	Se	Se	Se	Se	Se	++
S. aureus	Re	Se	Se	Re	Se	Se	Se	Se	Re	Se	++
S. aureus	Se	Re	Se	Se	Se	Se	Se	Se	Re	Se	++
S. aureus	Re	Re	Se	Se	Re	Se	Se	Se	Re	Se	++
S. aureus	Re	Se	Se	Se	Se	Se	Se	Se	Re	Re	++
S. aureus	Re	Se	Se	Se	Re	Se	Se	Se	Se	Se	++
S. aureus	Re	Se	Se	Se	Re	Se	Se	Se	Se	Re	++
E. coli	Re	Re	Se	Se	Se	Se	Re	Se	Se	Se	--
E. coli	Re	Re	Re	Re	Se	Se	Se	Se	Se	Se	--
E. coli	Re	Se	Se	Se	Se	I	Se	Se	Re	Se	--
E. coli	Re	Re	Se	Se	Se	Se	Se	Se	Re	Se	--
E. coli	Re	Re	Se	Se	Se	Se	Se	Se	Re	Se	--
E. coli	Se	Re	Se	Se	Se	Se	Se	Se	Se	Se	--
E. coli	Re	Se	Re	Re	Se	Se	Se	Se	Se	Se	--
E. coli	Se	Re	Re	Re	Se	Se	Se	Se	Se	Se	--
E. coli	Se	Re	Se	Se	Se	Se	Se	Se	Se	Se	--
E. coli	Se	Re	Se	Re	Se	Se	Se	Se	Se	Se	--
S. typhi	Se	Re	Se	Se	Se	Se	Se	Re	Se	Re	+
S. typhi	Re	Re	Se	Se	Se	Se	Se	Se	Se	Se	+

S. typhi	Se	Re	Se	Se	Se	Se	Se	Se	Se	Se	+
S. typhi	Se	Re	Se	Se	Se	Se	Se	Se	Se	Se	+

Key: Se, Sensitive. Re, Resistant. +, 10-15 mm. ++, 15-20 mm. --, lessthan 10 mm zone of inhibition.

Due to natural habitat of molds diverse groups of fungal strains inhabit in soil which made it second largest microbial population in the soil micro-flora. Organic compounds rich soil harbor much proportion of fungal strains because of its nutrients requirements, majority of fungi exist in the soil as saprophytes. Physical and chemical parameters of soil usually change the population of fungi [174]. Structurally novel compounds had been extracted from soil fungi which became the source of many available antibiotics in the market. Six out of 20 prescribed medications were extracted from fungi [175].

In the present study new fungal isolate primarily identified as *A. ochraceus* capable of producing bioactive metabolites against pathogenic bacteria. Isolated pathogenic bacteria were resistant to two or more than two available antibiotic disc recommended for sensitivity assay. *A. ochraceus* revealed significant results against all tested pathogenic bacteria which showed possible solution to curb existing resistance to antibiotics. In another study *A. ochraceus* "(CL 41582)" was tested against multi drugs resistant bacteria which inhibited *S. aureus*, *S. pyogenes* and *E. faecalis* [176]. In the present findings we used (Cefotaxime, Cephradine, Levofloxacin, Sparfloxacin, Linezolid, Vancomycin, Teicoplanin, Fusidic acid, Cotrimaxazole, Chloramphenicol) antibiotics and 8 mm diameter agar discs having diffused bioactive metabolites of *A. ochraceus* were gently placed on the prepared lawn of isolated pathogenic bacteria. Ten to fifteen mm zone of inhibition was observed against Gram negative bacterial pathogens except *E. coli* which revealed less than 10 mm zone of inhibition. Fifteen to twenty mm zone of inhibition was found out against Gram positive bacteria.

Our findings are in consistent with the previous study in which a new compound was extracted from *A. ochraceus* had antimicrobial activities against MDR *S. aureus* which

showed resistance towards 8 different antibiotics and multi drugs resistant *S. pyogen* and *E. faecalis*. Isolated compound tested against clinical isolate of *E. coli* showed no activity [176]. Another study conducted on molds secondary metabolites, seven fungal strains were tested against 22 Gram positive and Gram negative clinical isolates which showed antibacterial activities against indicator bacterial strains. Extracted antibiotics were more potent against Gram positive clinical isolates than Gram negative isolates [177]. In the present study we used 45 clinical bacterial isolates, and isolated 9 fungal strains only one strain was found active against tested bacterial isolates primarily identified as *A. ochraceus*. Potent soil isolate was more active against Gram positive bacteria than Gram negative bacteria.

3.7 Isolation of actinomycetes for antimicrobial activity

In the last few decades worldwide several unexplored habitats were extensively screened for bioactive compounds producing actinomycetes [178]. A study conducted in Baida area of Jordan *Streptomyces* strains isolated from soil active against antibiotic resistant bacteria indicating novel bioactive metabolites production [179].

Selection of unexplored diverse range of habitats in KPK for soil sampling was targeted to isolate actinomycetes flora active against wide range of indicator bacterial strains. Total 220 actinomycetes were isolated. In the primary screening for antibiotic production 35 actinomycetes inhibit the growth of one or more than one tested isolated pathogenic bacteria (Table 11). For further confirmation target objectives was achieved by successful screening of 27 bioactive compounds producing actinomycetes (12.27%), active against some Gram positive and Gram negative antibiotic resistant pathogenic bacteria and "ATCC" bacterial strains in the secondary screening. Out of these 27 antibiotic producing

actinomycetes 14 actinomycetes were isolated from soil samples, collected from unexplored areas of KPK 7, 5, 6, 4, 9, 4 and 8 actinomycetes were from Miaadam hilly areas and from Matta plain agricultural land of Swat valley while from Peer baba ghazi khanay plain agriculture land, Peer abhy plain areas, Daggar hilly areas (forest), Cheena asad abad bar kalay and from Gagarra nonagricultural land of Bunir valley respectively. While 13 antibiotic producing actinomycetes were isolated from different areas of district Mansehra, Dir lower and Dir upper regions (table 1). Out of 220 isolated actinomycetes, 27 were active against some Gram positive and some Gram negative pathogenic bacteria in secondary screening. Fourteen isolated actinomycetes (RMN1 to RMN14) had significant antibacterial activities against tested bacterial strains.

a b c d

Figure 15. a). Isolated actinomycetes on nutrient agar media using 10^{-2} diluted sample in the presence of antifungal and antibacterial compounds. b). Isolated actinomycetes on actinomycetes agar media using 10^{-5} diluted soil sample in the presence of antibacterial and antifungal compounds. c). pure culture of selected actinomycetes RMN6 on nutrient agar media d). Antibacterial activities of Isolate RMN 6 actinomycete tested against *Micrococcus luteus* ATCC 10240 through agar well diffusion assay.

Isolation media were actinomycetes isolation agar (AIA), starch casein agar (SCA) and nutrient agar for isolation of actinomycetes from soil. All isolated actinomycetes were primarily identified morphologically and microscopically as actinomycetes.

3.7.1 Primary screening

Screening of potent producers of antimicrobial compounds was pointed out through cross streak agar method against all isolated human pathogenic bacteria. Thirty five actinomycetes were found active against at least one indicator strain which indicated that 15.90% isolated strains of actinomycetes had bioactive potential against human (MDR) bacterial pathogens. Some producing strains were found active against all pathogenic strains tested while some have narrow spectrum activity active against only Gram positive or only against Gram negative isolated pathogens (Table 11).

Table 11. Antimicrobial activities of isolated actinomycetes against isolated pathogenic bacteria

Antibiotic Producing Actinomycetes	*E.Coli ESBL producing*	*P. Aerogi nosa*	*S. aureus* (MRSA)	*Enterbacter spp*	*S. Typhi*	*Streptococcus spp*	*P. vulgaris*	*P. mirabilus*	*Klebsiella spp*	*Enterococcus spp.*
RMN1	-	-	+	++	-	-	-	-	-	-
RMN2	+	+	+	+	+	+	-	-	+	+
RMN3	-	+	+	-	-	-	-	-	-	+
RMN4	-	-	-	-	+	-	-	+	-	-
RMN5	+	-	-	-	-	-	-	-	-	-
RMN6	+	+	+	+	+	+	+	+	+	+
RMN7	-	-	-	-	+	+	+	-	-	-
RMN8	+	+	+	+	+	+	+	+	+	+
RMN9	+	+	+	-	+	-	+	+	-	-
RMN10	+	+	+	+	+	-	-	-	-	-
RMN11	-	-	+	-	-	+	-	-	-	-
RMN12	-	-	+	-	-	-	-	-	+	+
RMN13	-	-	+	-	-	-	-	-	-	+
RMN14	+	+	+	+	+	+	+	+	+	+
RMN15	-	-	-	-	-	-	+	+	-	-
RMN16	-	-	-	+	-	-	+	-	-	-
RMN17	-	-	+	-	-	-	-	-	-	-
RMN18	+	+	+	-	-	-	-	+	+	-

RMN19	-	-	+	-	-	-	-	-	-	-
RMN20	+	-	+	-	-	+	-	-	-	-
RMN21	-	-	-	-	-	-	+	-	-	-
RMN22	+	+	-	-	-	-	-	-	-	+
RMN23	-	-	+	+	-	-	-	-	-	-
RMN24	-	-	-	-	-	+	+	-	-	-
RMN25	-	-	-	+	+	+	-	-	-	+
RMN26	-	-	-	-	-	-	-	+	+	-
RMN27	-	-	-	-	+	+	-	+	+	+
RMN28	-	-	+	-	-	-	-	-	-	-
RMN29	+	+	-	-	-	-	-	-	-	-
RMN30	-	-	+	+	+	-	-	-	+	+
RMN31	-	+	-	-	-	-	-	-	-	+
RMN32	-	-	+	+	-	-	-	-	+	-
RMN33	+	+	+	+	-	-	-	+	+	-
RMN34	-	-	-	+	-	-	+	-	-	-
RMN35	-	-	-	-	+	+	+	-	-	+

3.7.2 Secondary screening

Furthermore primarily screened antibiotic producers were confirmed through agar well diffusion assay excellent results were observed against selected indicator "ATCC" and isolated selected human pathogenic bacterial strains. Inhibitory activity towards "ATCC" bacterial culture and human pathogenic bacteria was at the range of 9 to 25 mm zone of inhibition. RMN 4 isolate was found active against only Gram positive bacterial strain, while RMN6 have most potent activity against all tested bacterial indicator strains which was at the range of 11 to 25 mm zone of inhibition against *P. aeroginosa* ATCC 27553 and *M. luteus* ATCC 10240, While against pathogenic bacterial isolates 13, 15, 18 and 23 mm zone of inhibition was recorded against *S. typhi*, *E. coli*, *P. aeroginosa* and *S. aureus* respectively. RMN7 isolate have bioactive potential against *M. leuteu* ATCC 10240 and *S. typhi* (clinical isolate) were 11 and 10 mm zone of inhibition. Actinomycetes RMN 8 have broad spectrum activity at the range of 11 to 19 mm zone of inhibition against *E. coli* ATCC 25922 and *S. aureus* (Table 12).

Table 12. Antimicrobial activity of primarily screened active actinomycetes against various bacteria by agar well diffusion method, secondary screening.

Activity against *ATCC* culture						activity against human pathogens				
Test bacteria zone of inhibition in mm										
Cod of isolates	*E.coli 25922*	*P.aeruginosa 27553*	*S.aureus 25923*	*K.pneumonia* 700603	*E.faecalis* 29212	*M.luteA TCC 10240*	*P.aeruginosaMDR*	*S.aureus MRSA*	*E.coli MDR*	*S.typhi MDR*
RMN1	0	0	14	10	11	15	0	13	0	0
RMN2	10	12	17	16	10	19	14	15	0	13
RMN3	0	0	12	0	0	0	10	10	0	0
RMN4	0	0	15	0	0	13	0	0	0	0
RMN5	13	0	0	0	0	0	0	0	0	0
RMN6	14	13	19	16	11	25	18	23	15	13
RMN7	0	0	0	0	0	11	0	0	0	10
RMN8	11	10	15	13	9	16	12	19	10	0
RMN9	0	0	12	0	0	17	13	14	12	13
RMN10	11	9	13	14	0	18	12	11	0	11
RMN11	0	0	0	11	12	11	0	0	0	0
RMN12	0	0	14	12	0	18	0	0	0	0
RMN13	0	0	12	0	0	13	0	0	0	0
RMN14	10	11	18	10	9	15	13	0	0	0

3.8 Cultural characteristics of selected isolates after secondary screening

Culture characteristics were observed on different media were, soluble pigments, aerial and substrate mycelium, color of aerial and substrate mycelium and other features (Table 13).

Morphological characteristics of soil isolate revealed filamentous growth on International *Streptomyces* project (ISP) media. Microscopic observations at 100X magnification viewing prepared slide after Gram staining revealed that isolated strains were filamentous rod shaped Gram positive. Macroscopic characteristics of colony morphology and growth of aerial hyphae and spore development showed similar characteristics with *Streptomyces*. Isolated Strains were designated as *Streptomyces* spp. compared with Berges Manuals [180-181].

Table 13. Culture characteristics of actinomycetes on different ISP media

Media	Culture, Characteristics	RMN1	RMN2	RMN3	RMN4	RMN5	RMN6	RMN7	RMN8
ISP1	Gr	A/growth	G/growth	H/growth	H/growth	Poor	Good	A/growth	A/growth
	AMy	Pink	White	B/brown	White	White	White	White	Milky
	SMy	Pink	White	B/brown	White	White	White	White	Milky
	SPe	No	No	No	No	No	No	Pinkish	No
	Sp	Pink	White	B/brown	White	No	White	White	Milky
ISP2	Gr	Good	H/growth	A/growth	A/growth	A/growth	Good	H/growth	A/growth
	AMy	white	White	Light/green	White	Green	Milky	White	Light/grey
	SMy	white	White	Light/green	White	Green	Milky	White	Light/grey
	SPe	No	No	No	No	No	No	No	No
	Sp	C/white	Grey	Light/green	White	Green	Grey	Grey	Grey
ISP3	Gr	H/growth	H/growth	H/growth	H/growth	H/growth	H/growth	H/growth	H/growth
	AMy	Milky	Grey	Green	Reddish	White	Black	White	Grey
	SMy	White	Grey	White	Red	White	Black	White	Grey
	SPe	No	No	No	No	No	No	No	No
	Sp	Pinkish	Grey	Green	Pink	Green	Black	Green	Grey
ISP4	Gr	Good	V good	V good	V good	V good	V good	Good	V good
	AMy	Brown	White	P/white	P/white	W/green	B/black	Yellow	W/green
	SMy	Brown	White	P/white	P/white	W/green	B/black	L/yellow	W/green
	SPe	No	No	No	No	No	No	Yellow	No
	Sp	brown	Brown	P/white	P/white	Green	B/black	Yellow	Green
ISP5	Gr	Poor	V good	V good	Poor	Good	Good	V good	V good
	AMy	White	White	M/cream	White	Green	White	M/cream	White
	SMy	White	White	W/green	White	Green	White	M/cream	White
	SPe	No	No	No	No	No	No	No	No
	Sp	C/White	White	Grey	White	W/green	Grey	Grey	White
ISP7	Gr	V good	V good	V good	V good	V good	Good	Good	V good
	AMy	Pink	Peach	W/green	W/red	Green	Cream	Grey	P/white
	SMy	White	Peach	W/green	White	Green	Cream	Grey	P/white
	SPe	No	No	No	No	No	No	No	No
	Sp	Pink	Peach	Green	White	W/green	White	Grey	P/white

Key: Gr=Growth, AMy=Aerial mycelium, SMy=Substrate mycelium, SPe=Soluble pigments, Sp=Sporulation, V=Very, W=White, P=pinkish, C=Creamish, G=good, H=heavy, A=abundant, B=Black.

3.9 Biochemical characterization of selected actinomycetes

Biochemical tests were conducted for 8 isolated actinomycetes (RMN1 to RMN8). RMN6 showed significant antimicrobial activities. Biochemical results of RMN6 revealed brown pigments on the prepared slants of Waksman media for 96 hours incubation at 28 C^0 brown melanin pigments formation were observed intermittently during incubation period. While for nitrate reduction test was positive in organic nitrate broth and red pink color was observed when Naphthalene solution (solution A) and sulfonilic acid solution (solution B) was added after one week incubation period at 28 ^{0}C. For proteolytic activity skimmed milk already pasteurized was utilized by actinomycetes RMN6 and acidic reaction was observed. Starch agar medium was used for hydrolysis of starch by the action of amylolytic enzymes secreted by soil isolate RMN6. By adding iodine solution in the already cultured slants of starch agar medium color changed indicates positive results. Nutrient gelatin medium inoculated through stab of isolate and incubated for two weeks at 28 ^{0}C nutrient gelatin medium was liquefied by RMN6 by extracellular enzyme secretion indicated positive results. Carbohydrate utilization agar ISP 9 was prepared along with bromocresole dye inoculated with 1 ml isolate RMN6 in the sterile petri plates. Discs having 3% of carbon sources were starch, dextrose, sucrose, maltose and lactose were placed gently on the medium surface and incubated at 28 C^0 for one week. Purple yellow colors around the disc of glucose indicate positive result for the utilization of glucose in the presence of oxygen. Inoculated glucose nutrient broth media incubated for two weeks at 28 C^0 revealed blue to yellow color production positive for acid production. Glucose broth media already inoculated was observed for color change after every 24 hrs. Prepared slants of hydrogen sulfide production media was inoculated

and incubated for one week at 37 C^0 rotten egg smell and medium color changed to black indicate positive result. Biochemical results of selected actinomycetes revealed that they belong to genera *Streptomyces* and designated as *Streptomyces spp*. RMN. Almost similar biochemical characteristics were also studied for other tested *Streptomyces spp.* (Table 14).

Table 14. Biochemical characteristics of isolated actinomycetes

Soil isolates	Melanin productio-n	Nitrate Reduct-ion	Proteolytic properties /milk coagulation	Liquefi cation of Gelatin	Hydrol-ysis of Starch	Carbon assimil-ation	Acid Produ -ction	Produ-ction of H_2S
RMN1	Good growth only	+ive	+ive	-ive	-ive	Glucose	-ive	+ive
RMN2	Growth without pigments	-ive	+ive/acidic reaction	+ive	+ive	Glucose	+ive	+ive
RMN3	Good growth only	+ive	+ive/slight acidic reaction	+ive	+ive	Lactose	+ive	-ive
RMN4	Light growth only	+ive	+ive/acidic reaction	+ive	+ive	Lactose	+ive	+ive
RMN5	Abundant growth only	+ive	Not clear acidic reaction	+ive	+ive	Lactose	+ive	-ive
RMN6	Brown pigment	+ive	Acidic reaction	+ive	+ive	Glucose	+ive	+ive
RMN7	Growth only	+ive	-ive	-ive	+ive	Lactose	-ive	-ive
RMN8	Growth only	+ive	+ive/clear acidic reaction	+ive	+ive	Lactose	+ive	+ive

3.10 Parameters optimization studies for *Streptomyces* RMN6 spp.

Maximum activity was observed against *Micrococcus luteus* and *Staphylococcus aureus* was 32 mm and 28 mm zone of inhibition which was optimized at pH 8, 144 hours incubation period, 29 ^{o}C, 160 rpm speed of shaking incubator and 7.5% inoculum size (Table 15-18).

3.10.1 Incubation Temperature and pH

The pH effects the growth and production of secondary metabolites during batch fermentation process. The H^{+} or ^{-}OH ion concentration may have direct lethal or beneficial effect on the vegetative cell or have indirectly effect by changing the degree of substances dissociation in the medium [182]. The effect of different pH studied was at the range of 5, 6, 7, 8 and 9. The effect of pH from 5 to 9 revealed that the antimicrobial activity increased and attained maximum with gradual increase of pH from 6 to 8. No activity was observed at pH 5 against both indicator strains. Further increase of pH to 9, antibacterial activity decreases against indicator strains. Best activity was observed at pH 8. The effect of pH indicates that change in pH profoundly affect bioactive metabolites production in production media. Initial incubation temperature ranges was adjusted from 25 to 37 ^{0}C. Antibacterial activities was increases from 25 to 29 C^{0} and then decreases up to 37 ^{0}C. Optimum activity was observed at 29 C^{0} (Table 15).

Table 15. Optimization of temperature and pH

Temperature optimization	Zone of inhibition in (mm)		pH optimization	Zone of inhibition in (mm)	
Temperature C^0	*S. aureus* MRSA	*M. luteus 10240*	pH	*S. aureus MRSA*	*M. luteus 10240*
25	15	21	5	0	0
29	**25**	**29**	6	12	13
31	22	24	7	18	21
34	14	20	**8**	**23**	**27**
37	0	13	9	20	26

3.10.1.2 Glucose concentration and incubation period

The glucose concentration from 1 to 6% was investigated for maximum production of antibacterial metabolites. Each concentration of glucose was added to the production medium for maximum production of metabolites. Results indicate that 3% glucose concentration was optimum for maximum production of metabolites and exceeding the 3% glucose concentration decreases the antimicrobial metabolite production. The batch fermentation was carried out for 8 days. Initially no activity was observed after 24 hours of incubation period then activity was recorded after 48 to 192 hours of incubation. Maximum antibacterial activity was observed after 144 hours of incubation (Table 16). Decrease in antimicrobial activity after 144 hours of incubation might be due to the depletion of nutrients and accumulation of toxic nitrogenous waste products in the medium.

Table 16. Optimization of D-glucose concentration and incubation period

D-glucose conc. optimization	Zone of inhibition in (mm)		Incubation period optimization	Zone of inhibition in (mm)	
D-glucose conc %	*S. aureus* MRSA	*M. luteus* 10240	Incubation period	*S. aureus* MRSA	*M. luteus* 10240
1	20	21	24	0	0
2	22	24	48	0	11
3	**23**	**27**	72	13	15
4	21	23	96	16	17
5	18	22	120	20	22
6	13	15	**144**	**23**	**25**
			168	22	23
			192	19	21

3.10.1.3 Inoculum size and speed of shaking incubator

Inoculum size percentage volume/volume was 5 to 17.5% inoculum. The difference between two ranges of inoculums was 2.5%. Inoculum volume of 7.5% was the best inoculum size for the maximum growth and antibacterial activity by producing strain RMN6 against two indicator strains. Speed of orbital shaking incubator was adjusted at 100 to 200 revolutions per minute (rpm). Best results were noted at 160 rpm adjusted for batch fermentation (Table 17).

Table 17. Optimization of inoculum size and speed of shaking incubator

Optimization for size of inoculum	Zone of inhibition in (mm)		Optimization of rpm in shaking incubator	Zone of inhibition in (mm)	
Size of inoculum %	*S.aureus MRSA*	*M. luteus 10240*	Speed of shaking incubator in rpm	*S.aureus MRSA*	*M. luteus 10240*
5	25	26	100	22	24
7.5	**27**	**29**	120	23	26
10	23	27	140	25	27
12.5	22	24	**160**	**26**	**29**
15	22	23	180	24	28
17.5	20	21	200	24	26

Optimization parameters adjusted at optimized values revealed that selected actinomycetes isolate *Streptomyces RMN6* have maximum antimicrobial activity against *M. luteus* ATCC 10240 (32 mm) and *S. aureus* (MRSA clinical isolate) (28 mm) zone of inhibition through agar well diffusion assay (Table 18).

Table 18. Antimicrobial activity of isolate RMN6 at optimized parameters against *Micrococcus luteus* and *Staphylococcus aureus*

Indicator strains	pH 8	Temperature 29 ^{0}C	Inoculum size 7.5%	Speed of shaking incubator 160 rpm	Incubation period 144 hours	D-Glucose concentration 3%
Micrococcus luteus ATCC 10240	**32 mm**					
Staphylococcus aureus (MRSA)	**28 mm**					

Due to the lake of education and proper training and health regulations for sale of antibiotics without prescription, antibiotic resistance increases especially in KPK at alarming rate especially Methicillin resistant *Staphylococcus aureus* and Gram negative multi drugs resistant pathogenic bacteria (MDR). It is clear from our results that we have open option for large scale screening of soil for isolation of actinomycetes producing bioactive metabolites against resistant pathogens to develop novel antibiotics for control of resistant pathogenic bacteria.

In the last few decades several unexplored habitats in the world for isolation of bioactive compounds producing microorganisms were extensively screened [178]. A study conducted in Baida area of Jordan, *Streptomyces* strains isolated from soil active against antibiotic resistant bacteria [179]. Selection of unexplored diverse range of habitats in KPK for soil sampling was targeted to isolate actinomycetes flora active against wide range of indicator bacterial strains. Target objective was achieved by successful isolation of 14 bioactive compounds producing actinomycetes active against some Gram positive and Gram negative antibiotic resistant pathogenic bacteria and ATCC bacterial strains. Selected Actinomycetes isolates (RMN1-8) were characterized biochemically and morphologically and designated as *Streptomyces* RMN spp. which was potent producer of bioactive metabolites confirmed in secondary screening. RMN6 isolate had potent activities against all tested indicator bacterial strains. Highest activity was observed against *M. leuteus* ATCC 10240 and *S. aureus* MRSA. Research investigations carried out in our study also correlates with another study conducted in Turkey in which actinomycetes were isolated from 11 soil samples collected from terrestrial environment. Isolated *Streptomyces* had antibacterial activity against 7 indicator bacterial strains

including multi drugs resistant bacteria. Five isolated *Streptomyces* had potent activities against *Methicillin resistant Staphylococcus aureus* [110]. In this research study soil samples were collected from diverse range of habitats in KPK, actinomycetes isolated showed broad spectrum as well as narrow spectrum activities against tested bacteria. It is obvious from our results that the soil micro flora exhibit potential pool of diverse actinomycetes active against human pathogenic bacteria and American type cell culture. Morphologically different groups of actinomycetes revealed that the soil of collected sites previously unexplored for screening have high diverse groups of actinomycetes producing broad spectrum as well as narrow spectrum bio active secondary metabolites. Tests indicator bacteria were Gram positive as well as Gram negative bacteria both ATCC bacterial culture and fresh isolated human pathogenic strains isolated from hospitalized patients samples, identified morphologically and biochemically which were resistant to wide range of antibiotics tested through disc diffusion assay. Seven isolated actinomycetes namely RMN1, RMN2, RMN3, RMN6, RMN8, RMN9 and RMN10 produced secondary metabolites against methicillin resistant *S. aureus*. While isolate RMN2, RMN3, RMN6, RMN8, RMN9 and RMN10 results indicates broad spectrum activity against *E. coli, P. aeroginosa, S. typhi and S. aureus* as well as ATCC bacterial strains tested. *E. coli* causes urinary tract infection especially in females. *S. typhi* infection developed typhoid fever these pathogenic bacteria became resistant to wide range of antibiotics [183]. MRSA is the major cause of hospital acquired infection worldwide and is the leading cause of infections [184].

Optimization studies conducted for selected actinomycetes isolate RMN6 indicates best activities at pH 8 was 23 and 27 mm, 144 hours incubation period 23 and 25 mm, 29 C^0

25 and 29 mm, 7.5% inoculum size, 27 and 29 mm, 3% glucose concentration 23 and 27 mm and 160 rpm speed of shaking orbital incubator was 26 and 29 mm zones of inhibition was observed against *M. leuteus* and *S. aureus* (MRSA) respectively. While when all parameters were optimized 28 and 32 mm zones of inhibition was noted against indicator bacterial strains. Similar optimum range of pH was reported by [185], for the antimicrobial activity by *Streptomyces sp.* RUPA-08PR isolated from soil. The results of our research findings were also nearly correlates with another study for optimized parameters conducted for Amycolatopsis Alba var. nov. DVR D4 specie revealed that 2% D- glucose concentration, 4% malt extract, 5% inoculum size, 28 ^{o}C incubation temperature, 220 rpm of orbital shaker speed and 96 hours incubation period was suitable for maximum antimicrobial activities [186].

3.11 Thin layer chromatography

Four spots were developed on the TLC plates observed under UV light at 254 nm and also observed along with iodine vapors. Our results showed antimicrobial activities by two tested spotted compounds which showed synergistic activities of compounds against tested bacteria (Table 19).

Table 19. Solvent ratio (v/v) for thin layer chromatography

S.No	Solvent system	
1	Methanol : Acetic acid	8.4 : 1.0
2	Methanol : Chloroform	9.2 : 0.8
3	n butanol : Acetic acid : Water	6 : 2 : 1

3.12 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of extracted antibiotics was carried out for Gram positive and Gram negative isolated pathogenic bacteria. Our results revealed that extracted fraction of antibiotics had broad spectrum activities against tested bacteria. The minimum inhibitory concentration values were in the range of 110 to 135 µg/ml. Our results indicated excellent results against isolated pathogenic bacteria. Another study showed that crude extract were tested against Gram negative and Gram positive bacteria was in the test range of 0.039 to 5 mg/ml [187]. Our results also revealed consistency with another study conducted for the determination of minimum inhibitory concentration when pure compound extracted from *Streptomyces spp*. A4 against Gram positive and Gram negative pathogenic bacteria which revealed similar outcomes for tested bacteria (Table 20), [108, 131].

Table 20. Minimum inhibitory concentration (MIC) against different pathogenic bacteria

Indicator pathogenic bacteria	MIC in µg/ml
Staphylococcus aureus (MRSA)	115
Escherichia coli ESBL (producer)	135
Pseudomonas aeroginosa (MDR)	110
Salmonella typhi (MDR)	115

In brief outcomes, pathogenic bacterial strains isolated from hospitalized patient exhibited high frequency of resistance to multiple numbers of antibiotics, which showed worse situation in Pakistan and especially at KPK province. To control antibiotic resistance or at least to slow down resistance pattern against available antibiotics we need proper regulations at government level, to implement health regulatory laws, to create awareness in the public regarding the use and misuse of antibiotics through print and electronic media and to encourage researcher for the accumulation of antibiotic resistance data time by time and also to find new antimicrobials as a controlling efforts. We isolate potent *Streptomyces* strains and one *Aspergillus* strain from the soil of KPK which were effective against multi drugs resistant pathogenic bacteria. If such trends of research findings continue then we will be able to add novel type's antimicrobials in the pipe line of pharmaceutical industries at local level, which will be inexpensive, potent and will be able to export such type of drugs to other countries for the benefit of humanity.

Conclusion

Our study concludes that total (542) pathogenic bacteria were isolated from 1296 human samples; highly prevalent bacteria were *E. coli* (43.91%), *S. aureus* (29.88%) and *P. aeroginosa* (15.68%) in the hospitalized patients admitted at Lady Rading Hospital (LRH) Peshawar.

Furthermore, gender wise prevalence of *E. coli* infection in female (52.93%) was higher than male patients (47.03%). Majority of *E. coli* were isolated from urine followed by pus, HVS, blood and CSF.

Most potent antibiotics tested against *E. coli* were Imipenem (2.94%), Meropenem (3.7%) Cefoperazone / Sulbactam (7.5%) and Piperacillin/Tazobactam (8.4 %) resistance pattern, while less potent antibiotics were Cephradine (96.63%), Cefuroxime (85.29%), Cephtriaxone (78.15%), Ceftazidime (75.21%), Coamoxiclave (72.68%), Cefotaxime (70.58%), Nalidixic acid (68.48%) resistance pattern was found. Total fifteen ESBL producing *E. coli* were characterized.

Majority of *Staphylococcus aureus* pathogenic strains were isolated from pus followed by high vaginal swab (HVS) and blood samples. Infection from *S. aureus* was comparatively higher in female than male patients. Total (161) *S. aureus* pathogens were investigated for antibiotic sensitivity assay in which 86 strains were Methicillin resistant *S. aureus* (MRSA). Antibiogram showed that most potent antibiotics resistance pattern were Vancomycin (0%), Taecoplanin (0%), Linezolid (1.20%), Fusidic acid (8.60%), while higher resistance was noted against Cotrimixazol (94.19%), Klathromycin (71.42%), Levofloaxacin (66.45%), Sparfloaxacin (65.56%) and Ciprofloaxacin (65.21%).

Total (85) *P. aeroginosa* strains were isolated from hospitalized patients. Higher numbers of P. aeroginosa strains were isolated from pus followed by urine, blood, ear swab and HVS samples. Prevalence of *P. aeroginosa* in male (67.05%) was higher than female (32.94%) patients. Most potent antibiotics were Meropenem (7.05%) Imipenem (8.22 %), Piperacillin/Tazobactam (10.58 %), and Cefoperazone/Sulbactam (17.64%).

Total (220) actinomycetes were isolated from soil samples and identified morphologically, (35) actinomycetes were screened out in the primary screening against isolated pathogenic bacteria. Primarily screened actinomycetes for antibiotic producing abilities were further process in the secondary screening (27) actinomycetes were found active for antimicrobial activities against pathogenic bacteria. Fourteen actinomycetes had significant activities against tested bacteria. Eight most potent actinomycetes were further identified biochemically, belongs to *Streptomyces* genera. One most potent *Streptomyces* strain RMN6 optimized parameters for maximum production of antibiotics in batch fermentation were pH (8), (144) hours incubation period, (29) C^0, (7.5%) inoculum size, (3%) glucose concentration and (160) rpm speed of shaking orbital incubator against *Micrococcus luteus* (ATCC 10240) and *Staphylococcus aureus* (MRSA clinical isolate) was 32 mm and 28 mm zone of inhibition respectively.

Four spots were developed on the Thin Layer Chromatography (TLC) plates observed under UV light at 254 nm and also observed along with iodine vapors. Our results showed antimicrobial activities by two tested spotted compounds which revealed synergistic activities of compounds against tested bacteria. The minimum inhibitory concentration against *Staphylococcus aureus* (MRSA), *Escherichia coli* ESBL (producer), *Pseudomonas aeroginosa* (MDR) and *Salmonella typhi* (MDR) were (115),

(135), (110) and (115) μg/ml respectively. These compounds need further isolation and structural elucidation to develop potent drug for control of resistant pathogenic bacteria.

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Annexure II

Patient's enrollment proforma

Patient Name:

Age	Sex	Nature of specimen collected	Name of ward	Bed number	Medical complication	Duration of stay in hospital

Consent: I am agree to give sample (specimen) for investigation and research purpose.

Sign:_____

Annexure III

The following the compositions of the media used for strain preservation and morphological studies.

1. Actinomycetes isolation Agar (AIA)	Gms/Lt
Heart infusion broth	25.0
Casein enzymic hydrolysate	4.0
Yeast extract	5.0
Dextrose	5.0
Cysteine HCl	1.0
Soluble starch	1.0
Potassium phosphate	15.0
Ammonium phosphate	1.0
Magnesium sulphate	0.2
Calcium chloride	0.02
Agar	20.0
2. Carbon utilization agar (ISP-9)	
Ammonium phosphate	2.64
Monopotassium sulphate	2.38
Dipotassium sulphate	5.65
Magnesium sulphate	1.0
Copper sulphate	0.006
Ferrous sulphate	0.0011
Magnesium chloride	0.0079
Zinc sulphate	0.0015

Agar	15
pH 7	

3. Glucose nutrient broth

Meat extract	3.0
Peptone	5.0
Glucose	10.0
pH 7	

4. Glycerol-Asparagine Agar Medium (ISP-5)

L-asparagine (anhydrous)	1.0
Glycerol	10.0
K2HPO4	1.0
Trace salts solution	1.0 ml
Distilled water	1.0 ml
pH 7.2 to 7.4	

5. Hydrogen Sulphide Production Medium

Ferric ammonium nitrate	0.5
Dibasic potassium phosphate	1.0
Na2S2O4	0.02
Yeast extract	1.0
Agar	20
pH 7	

6. Inorganic salt – starch agar medium (ISP-4)

Solution I

Soluble starch – 10 gm was made into paste with the small amount of cold water and then the volume was made up to 500ml.

Solution II	
K2HPO4	1.09
NaCl	1.09
Ammonium sulphate	2.09
Calcium carbonate	2.09
Trace salt solution	1.0 ml
Distilled water	500 ml
pH 7.0 to 7.4	
Solution I & II were mixed	
7. Nutrient Gelatin	
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
pH 6.8	
8. Organic Nitrate Broth	
Peptone	0.5
Meat extract	0.3
KNO3	0.1
pH 7.0	
9. Peptone – Yeast Extract Iron Agar (ISP-1)	
Peptone	20.0
Ferric ammonium citrate	0.5
K2HPO4	1.0
Na2S2O35H2O	0.08
Yeast extract	1.0

Solution II	
K2HPO4	1.0
NaCl	1.0
Ammonium sulphate	2.0
Calcium carbonate	2.0
Trace salt solution	1.0 ml
Distilled water	500 ml
pH 7.0 to 7.4	
Solution I & II were mixed	
10. Nutrient Gelatin	
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
pH 6.8	
11. Organic Nitrate Broth	
Peptone	0.5
Meat extract	0.3
MHO3	0.1
pH 7.0	
12. Peptone – Yeast Extract Iron Agar (ISP-6)	
Peptone	20.0
Ferric ammonium citrate	0.5
K2HPO4	1.0
Na2S2O35H2O	0.08
Yeast extract	1.0

Agar	20.0
pH	7.0

13. Tyrosine Agar (ISP-7)

L-Tyrosine	0.5
Glycerol	15.0
L-Asparagine	1.0
K2 HPO4 – 7H2O	0.5
MgSO4 7H2O	0.5
NaCl	0.5
FeSO4 . 7H2O	0.5
Trace salts solution	1.0 ml

Distilled water 1 lit

Agar	20.0

pH 7.2

14. Waksman No.42 Medium

Yeast Extract	1.0
L.tyrosine	1.0
Sodium chloride	8.5
Agar	16.0

pH 6.8

15 KIA medium

Composition of KIA media:

Peptone, Lab-Lemco powder, yeast extract, sodium chloride, lactose, glucose (dextrose), ferric citrate, sodium thiosulphate, phenol red, agar 5.5 g/100 ml.

16 Composition of Potato dextrose agar (PDA).

Potatoes, infusion from	200.000
Dextrose	20.000
Agar	15.000
Final pH (at 25°C)	5.6±0.2

17 Composition of starch casein nitrate medium

Starch	10
NaCl	2
KNO3	2
K2HPO4	1
Trypton	0.3
MgSO4	0.1
CaCO3	0.1
FeSO4.7H2O	0.02
ZnSO4	0.15%
PH	7.2

18 Composition of nutrient agar

Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000
Final pH	7.4±0.2