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DETECTION OF CARBAPENEM RESISTANT METALLO-BETA LACTAMASES GENE AMONG *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED FROM CANCER PATIENTS

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ABSTRACT

The study was conducted for the detection of carbapenem resistant beta lactamase genes including VIM, and IMP in multi drug resistant *Pseudomonas aeruginosa* from cancer patients. These genes play the most significant role in multi drug resistance. In this study, we used the disc diffusion method for the determination of antibiotic resistance against six antibiotics and PCR technique for the detection of genes. The results showed that the *P. aeruginosa* isolates were resistant against Meropenem (MEM) 68%, Imipenem (IMP) 92%, Polymyxin B (PB) 64%, Gentamicin (CN) 60%, Tobramycin (TOB) 16% and Tazobactam (TZP) 12%. The PCR results showed that 82% isolates were positive for the VIM and 72% was positive for IMP genes. These results revealed high emergence of resistance in carbapenem of *P. aeruginosa* isolates studied. This is the first report showing detection of carbapenem resistant gene against *P. aeruginosa* in cancer patient from Pakistan. The study might be helpful in understanding the molecular basis of antibiotic resistance in *P. aeruginosa* from cancer patients.

Key words: METALLO-BETA, *PSEUDOMONAS AERUGINOSA,* CANCER

Introduction

Pseudomonas aeruginosa is very important specie of Genus *Pseudomonas* because it causes many infections. It is key member of the family *Pseudomonadaceae*. Under the microscope, it appears as straight chain or marginally bent in shape, *Pseudomonas* are aerobic in nature and they also have flagella for movement (Ahmad *et al.,* 2016). It is gram negative bacteria and is also non-fermenting. It is famous due to the property of causing the disease in patient which are already suffering from the diseases e.g., patient suffering from cancer, HIV patients and burn patients. Due to the infectious property of its morbidity and mortality occur (Mansour *et al*., 2013). Many times, the type produce a blue-green pigment pyocyanin; on the culture media so this property of *P. aeruginosa* is very important and significant in the identification. The bacteria are named *aeruginosa* due to the characteristic color of copper oxide (Bonomo *et al.,* 2006). In Hospitals and specifically in intensive care units, it is most common pathogen which cause the diseases in immune-compromised patient some time also cause death. It is known as superbug since the entry of antibiotics in clinical treatment. It has developed increasingly more sophisticated resistant mechanism against the antibiotics. So due to this property it is famous as "super bug" (Bashir *et al.,* 2011). Multi drug resistance strains are responsible for hospital acquired diseases, especially in the population at risk most commonly patient with cancer. MDR *P. aeruginosa* causes the major problems in cancer or cystic fibrosis patient (Chadha *et al.,* 2014). Due to the MDR much cancer type develops resistant against the chemotherapy drugs, it is the most important factor in the failure of

much type of chemotherapy drugs. It shows effect on a patient e.g. blood cancer, tumor, breast cancer lung and gastrointestinal tract cancer. Bacteremia due to the MDR bacteria is life threatening in to the cancer patient (Faghri *et al.,* 2014). Cancer also has the ability to develop resistance against the anti-cancer therapies. So, the prevalence of drug resistance cancer also is on the increase (Hassuna *et al.,* 2015). The awareness increases in the care of cancer patient. First time *P. aeruginosa* produced the metallo-β-lactamase was isolated from the breast cancer with VIM-7 gene which is known as Verona integron–encoded metallo-β-lactamase VIM family. Later on, from the same cancer research institute *P. aeruginosa* which produced VIM-2 was isolated from the cancer patient. In Mediterranean basin, the carbapenem resistance in *P. aeruginosa* due to VIM family is mostly common. *P. aeruginosa* produces NDM-1 gene was reported in Patient with leukemia in Italy. In Europe VIM is known as internationally successful XDR "high-risk clone" (Peymani *et al.,* 2014).

Pseudomonas aeruginosa is survived in all kind of environments. So that's why it's also famous due to its survival nature and it shows resistance against antibiotics and antiseptics especially in hospitals and specifically in intensive care units. It is most common pathogen which causes the diseases in immune-compromised patient some time also cause death. Due to the resistant against all available antibiotics, gram negative *P. aeruginosa* causes so many diseases in humans (Hammami *et al.,* 2011). It has practically demonstrated all known enzymic or mutational mechanism of bacterial resistance (Strateva *et al.,* 2009). When the bacteria show the resistance against the more than three antibiotics it is defined as MDR bacteria. It is resistant to many drugs so it is also come in to the category of Multi drug resistant bacteria (Hassuna *et al.,* 2015).

Centre for Disease Control and Prevention (CDC) stated the definition in current study the organism acquired resistance to at least one agent in three or more different antimicrobials types. It shows the resistant against the variety of antimicrobials mainly Aminoglycosides, Penicillin's, Carbapenem, Cephalosporin's and Quinolones (Mandsberg *et al.,* 2009). The infection rate varies from 6 to 14 per 10,000 patients admitted per year due to infection caused by *P. aeruginosa* which is multi drug resistance. The study conducted in Rome Italy in hospital reveals that the first time from the hematologic patient *P. aeruginosa* reported in 1992. After the first report the incidence rate is on increase from 8% to 17% in 1993 to 1999 related to nosocomial infection. The Mortality rate is higher the 20% due to infection of *P. aeruginosa* and it's on the increase when the infection occurs due to the MDR *P. aeruginosa* (Khan *et al.,* 2014).

Carbapenem is a β-lactam antibiotic which includes Imepenem, Meropenem, Doripenem and Ertapenem. This family of antibiotic has a broad-spectrum activity and in the serious infection of Multi drug resistant *P. aeruginosa* these antibiotics use for the treatment (Wolter *et al.*, 2009). Carbapenem is a metallo β-lactam antibiotic relatively stable. MBL is belonging to the structural classification of β-lactamases have an ability to efficiently hydrolyze all β-lactam (Yan *et al.,* 2001). Carbapenem are divided in to two types of families on molecular level on the basis of a serine production on the active site and those who have one zinc atom on the active site are known as metallo-carbapenemases which is also a type of MBL. The clinically significant type of carbapenemases is VIM, IMP and SPM. The VIM encoded by bla (VIM), The IMP encoded by bla (IMP) and the SPM encoded by bla (SPM). 14 different types of VIMs and 23 different types of IMPs are identified. Carbapenemases are classified in to the IMP, VIM, SPM, GIM, SIM, and DIM, AIM, KHM, NDM and KPC family. GIM are found on a class 1 integron and IMP is found on a class 3 integron (Picao *et al.,* 2009). Imepenem is act on a renal dehydro peptidase I and act in combination with cilastain. Meropenem is less efficient as compared to imepenem again gram-positive bacteria but on the other side Meropenem is more effective against the gram-negative bacteria (Meletis *et al.,* 2012). Impermeability is the main cause of resistant against carbapenem (Pitout *et al.,* 2005).

Materials and methods

This research was conducted in the period of six month. We collected 150 different types of samples e.g. blood samples, urine samples and nasal samples from the patients which were cancer diagnosed patient from Shaukat Khanum Memorial Hospital and Research Centre Lahore. After sampling, we appropriately labeled all the samples and theses samples are straight away transported to the research laboratory of the Faculty of Life Sciences, University of Central Punjab Lahore for further processing. After collection and immediately after transportation these samples were processed for further procedure. For the inoculation of samples nutrient agar was used which is firstly autoclaved and then all the samples were streak under sterile conditions. After labeling the samples these plates were placed in the incubator on a growth condition which is 24 hours at 37^{o}C. Then After 24 hour's Petri plates were checked. First of all, the growth of the samples was recognized on the basis of bacterial colony type, colony morphology and on the basis of macroscopically characteristics. After Macroscopic examination we processed the samples for gram's staining for Microscopic Examination. The gram staining is used to explore the microscopic characteristics of bacteria. After identification with the gram staining further some biochemical test was performed for confirmation of isolates with analytical profile index which is the fast technique for the classification of bacteria. Then we performed the oxidase test which was a confirmatory test for *Pseudomonas aeruginosa*. Oxidase is an enzyme which is produced by bacteria to use in bacterial transport chain. Tetramethyl-p-phenylenediamine reagent tends to oxidize by cytochrome c Oxidase and give the intuition of purple color under indophenols sign of positive test. No color production indicates the negative result. After confirmation with the biochemical test we processed our samples for Antimicrobial susceptibility Testing.

The antimicrobial susceptibility test is use for the evaluation of antibiotics efficacy out which specific antibiotic a specific bacterium is sensitive to. In this test many antibiotics are used like Meropenem (MEM), Imipenem (IMP), Polymyxin B (PB), Gentamicin (CN), Tobramycin (TOB), and Tazobactam (TZP). Different types of method are used for the evaluation of susceptibility in this research disk diffusion method was used. Disk diffusion method is most commonly used to conclude the resistance of bacteria to antibiotics in clinical laboratories. In this technique the impregnated antibiotic disc placed on a Muller-Hinton agar. After evaluating the resistance by disc diffusion method, we processed samples for DNA extraction For DNA extraction colonies of bacteria are used. Take one ml of distilled water in the test tube and add the colony of bacteria in it then boil for 10 minutes in the water bath. After boiling centrifuge the tube at 1000rpm for five minutes. For the purpose of PCR five micro litter of supernatant were used.

Primer used in PCR

1. Bla (VIM)

VIM-F GATGGTGTTTGGTCGCATA

VIM-R CGAATGCGCAGCACCAG

2. Bla (IMP)

IMP-F GAAGGCGTTTATGTTCATAC

IMP-R GTATGTTTCAAGAGTGATGC

After DNA Extraction PCR was performed on all the DNA samples extracted using the methods. Two micro liters of the DNA were mixed with 10 µl of pre-aliquoted ReddyLoad PCR Master Mix containing 1.25 units of Taq DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM (NH4)2SO4, 1.5 mM MgCl2, 0.01% (v/v) Tween 20, 0.2 mM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and 100 pmol of each of the primers. The expected sizes of PCR products for the two sets of primers were 445 and 401 base-pairs (bp). For primers, the PCR mixture was incubated for five minute at 95 °C as an initial denaturation step, followed by initial DNA release and denaturation at 94˚C for 5 min, followed by 36 cycles of 94˚C for 30 s, 52˚C for 40 s and 72˚C for 50 s, followed by a single, final, elongation step at 72˚C for 5 min.

For the DNA size separation, the typical lab procedure is gel electrophoresis for the purification and visualization. By use of gel electrophoresis we can determine the accurate length of the DNA by comparing with DNA ladder consisting of a fragment of DNA with known lengths. For gel electrophoresis we prepared the 2% agarose for the preparation of 2% gel add 4.0-gram agarose in the 200 ml of 1X TBE buffer in a 600 ml beaker. After adding the agarose heat the beaker on a hot plate until the agarose is dissolved. Confirm that the agarose is dissolved. Add 15ul ethidium bromide in it. Then poured it in to the tray. After solidification load the samples in to the wells. After loading the samples turn on the electric field at 120 volts for 30 min

Results and discussion

Sample Collection

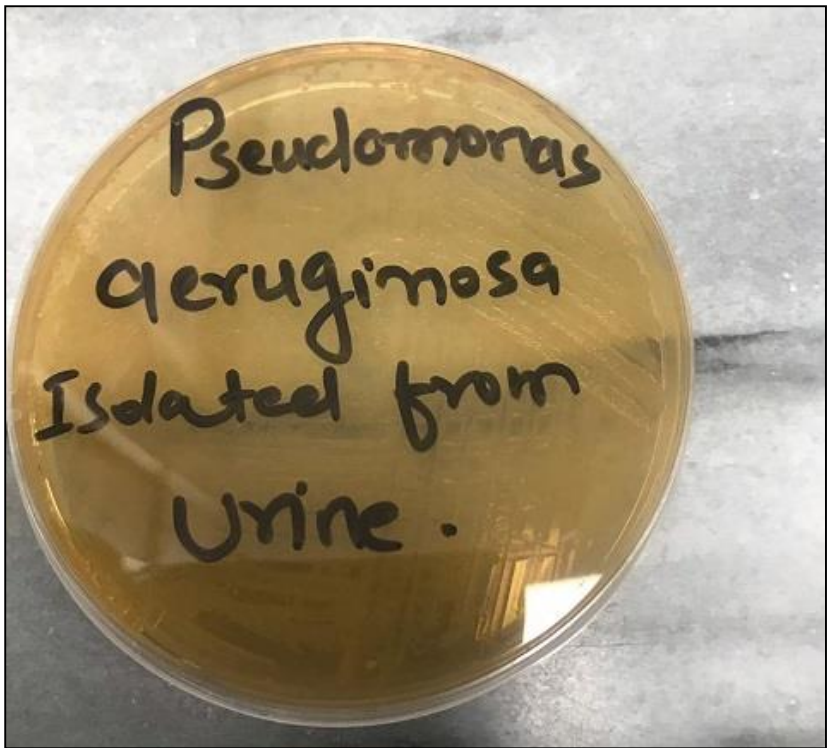
A total of 150 samples of Cancer patients were collected from different wards of the Shaukat Khanum Memorial Hospital and Research Centre for the present research. 150 samples include blood samples, urine samples and Nasal samples.

On the basis of lab tests of Blood, Urine and Nasal it was found that all of the 150 samples were positive for different pathogens. Out of these 150 samples we separate the 50 positive samples of *P. aeruginosa.*

And out of these 50 samples twenty-five blood samples are positive with *Pseudomonas aeruginosa* 10 urine samples are positive with *Pseudomonas aeruginosa* and 15 nasal samples are positive with *P. aeruginosa.*

Isolation and Identification

Pseudomonas aeruginosa grown on Nutrient agar and CLED agar show Colonies are surrounded by bluish green coloration -hemolytic colonies β . Pale yellow colonies i.e. non lactose fermenters On CLED media pigments are more obvious *Pseudomonas aeruginosa* able to grow at temperatures as high as 42 degrees. *Pseudomonas* growth on nutrient media and on CLED media showed in Fig:



Gram's staining

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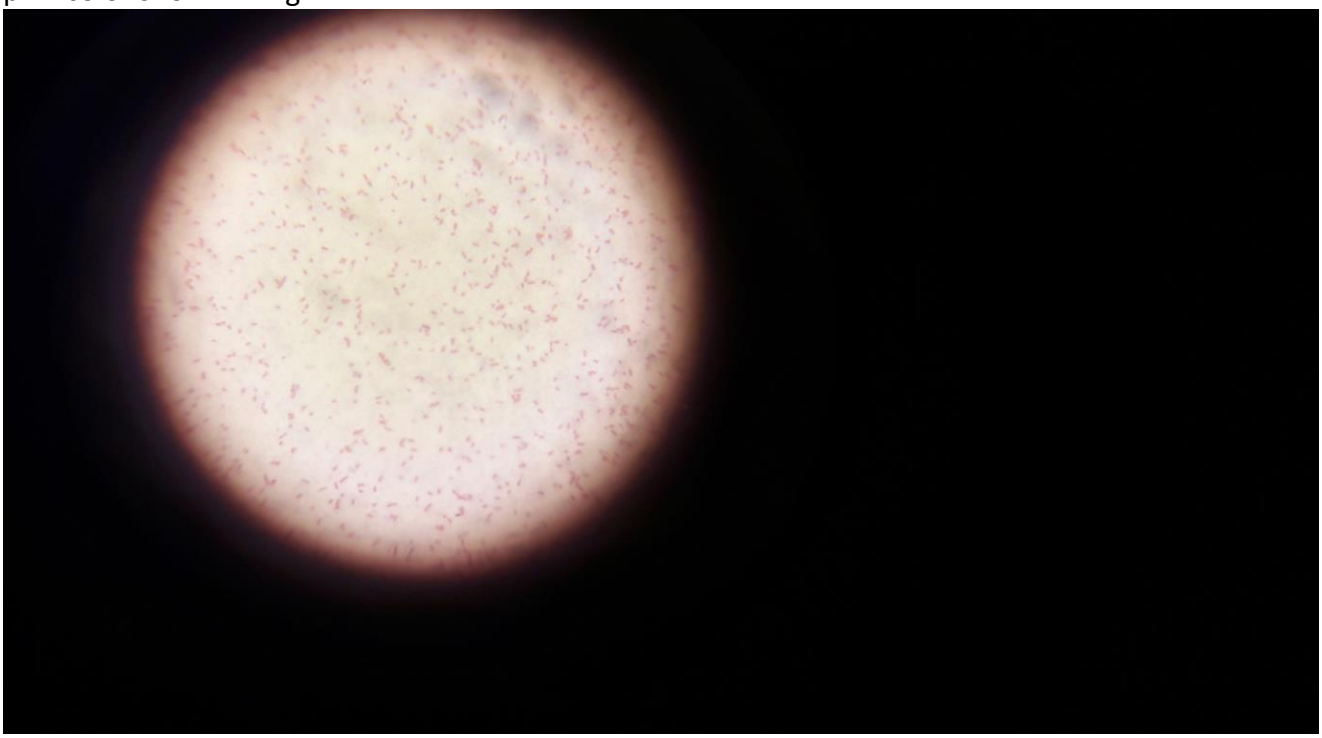
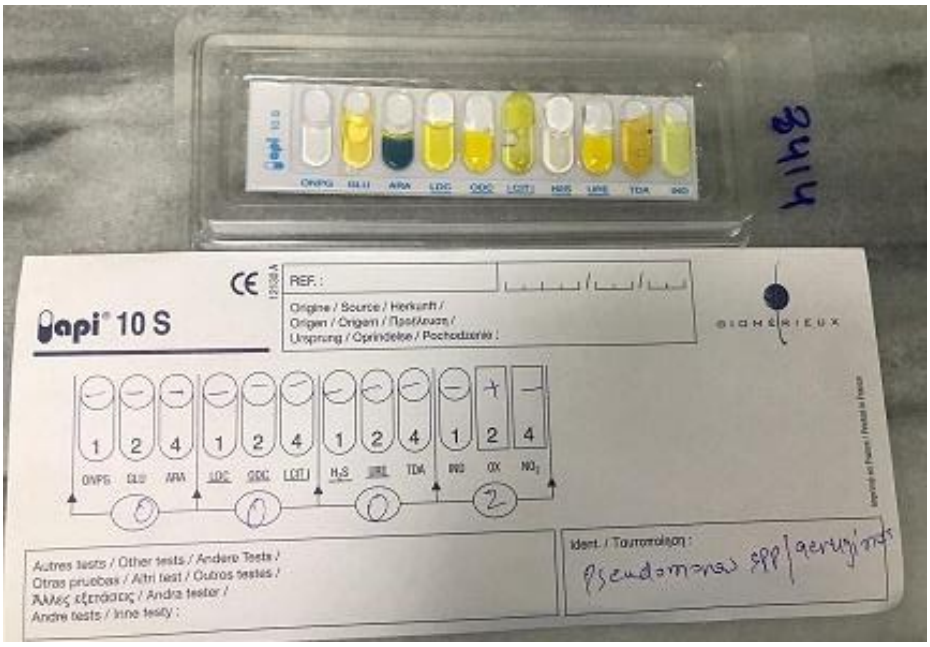
Pseudomonas aeruginosa is rod-shaped gram-negative bacteria. *Pseudomonas aeruginosa* are motile with the help of flagella and shows the pink color under the microscope. Gram negative rods in pink color shown in Fig:



Biochemical Characterization

Biochemical tests were performed for the authentication and characterization of the isolates. Biochemical tests were performed with the help of Analytical profile index to confirm the *Pseudomonas aeruginosa*.



Oxidase Test

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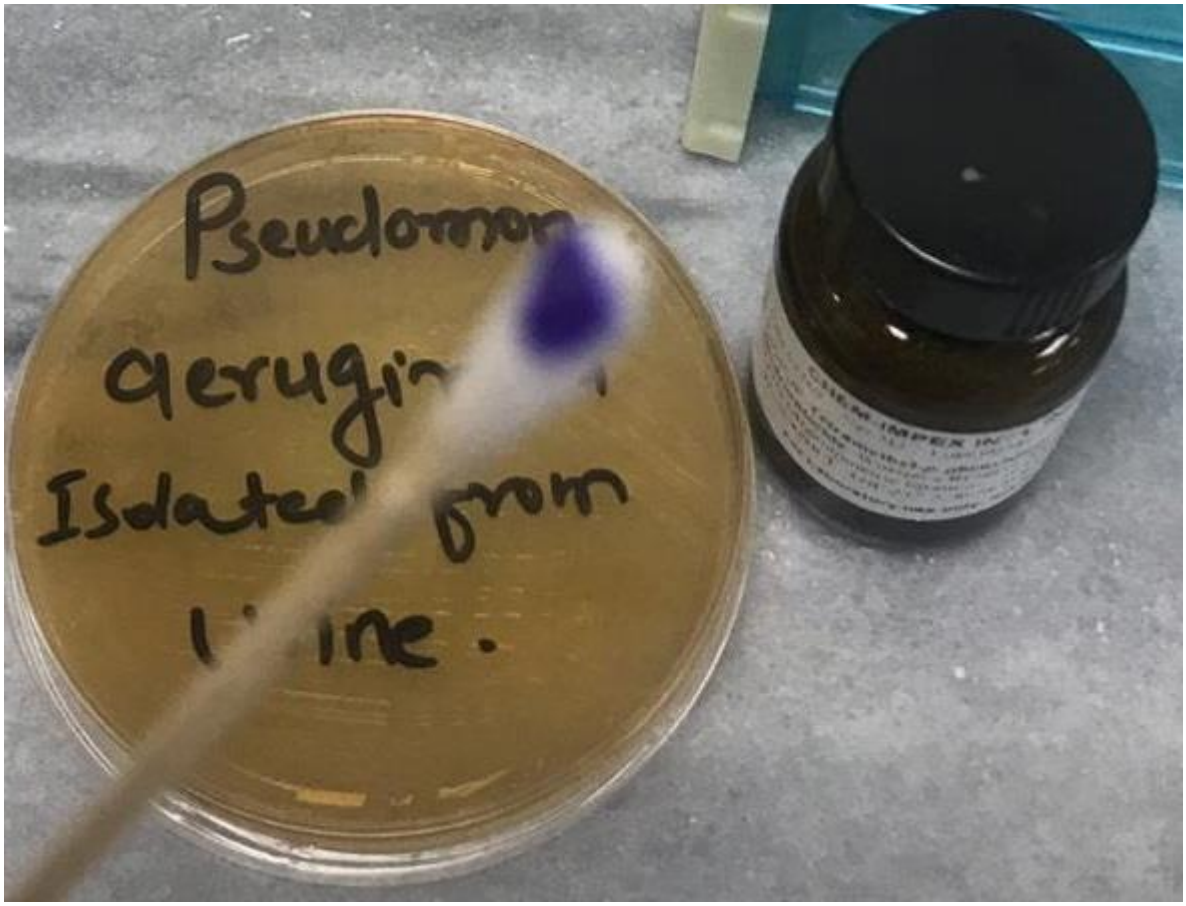
Oxidase is an enzyme produce by the bacteria the test is used to distinguish the bacteria on the basis of oxidase production. Oxidase is confirmatory test for *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* oxidase positive as shown in Fig:



Percentage on basis of Gender

Percentage of *Pseudomonas aeruginosa* on the basis of gender shows different variation as shown in Table. I.

Table: I Percentage on the basis of Gender

Gender	Positive Samples	Percentage
Men	24	48%
Women	26	52%

Percentage on the Basis of Age Group

Percentage of *P. aeruginosa* is varies in different age group as shown in Table. II.

Table II: Percentage on the basis of age group

Age Group	Samples	Percentage
1-10	7	14%
11-20	8	16%
21-30	15	30%
31-40	10	20%
41-50	10	20%

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests are utilized to figure out which particular antibiotics a specific microscopic organisms or growth is sensitive to. In this test various antimicrobial drugs like Meropenem (MEM), Imipenem (IMP), Polymyxin B (PB), Gentamicin (CN), Tobramycin (TOB), and Tazobactam (TZP) are used to cure patient's infection. The method used in present research for this purpose was disk diffusion method. In the results different antibiotic shows the different sensitive or resistant pattern as shown in Fig:

In this test *Pseudomonas aeruginosa* shows antimicrobial drugs resistant against Meropenem (MEM) 68%, Imipenem (IMP) 92%, Polymyxin B (PB) 64%, Gentamicin (CN) 60%, Tobramycin (TOB) 16%, Tazobactam (TZP) 12% as shown in Table:

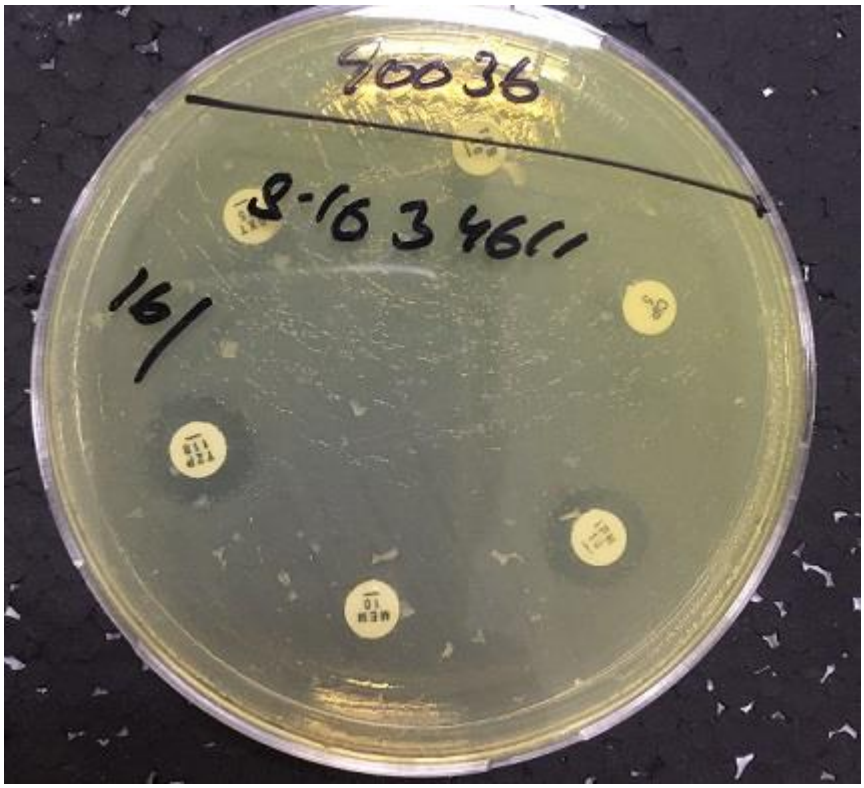


Fig: IMP and ME and TZP and TOP Resistance

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Fig: IMP and TZP and MEM and TOB Resistance

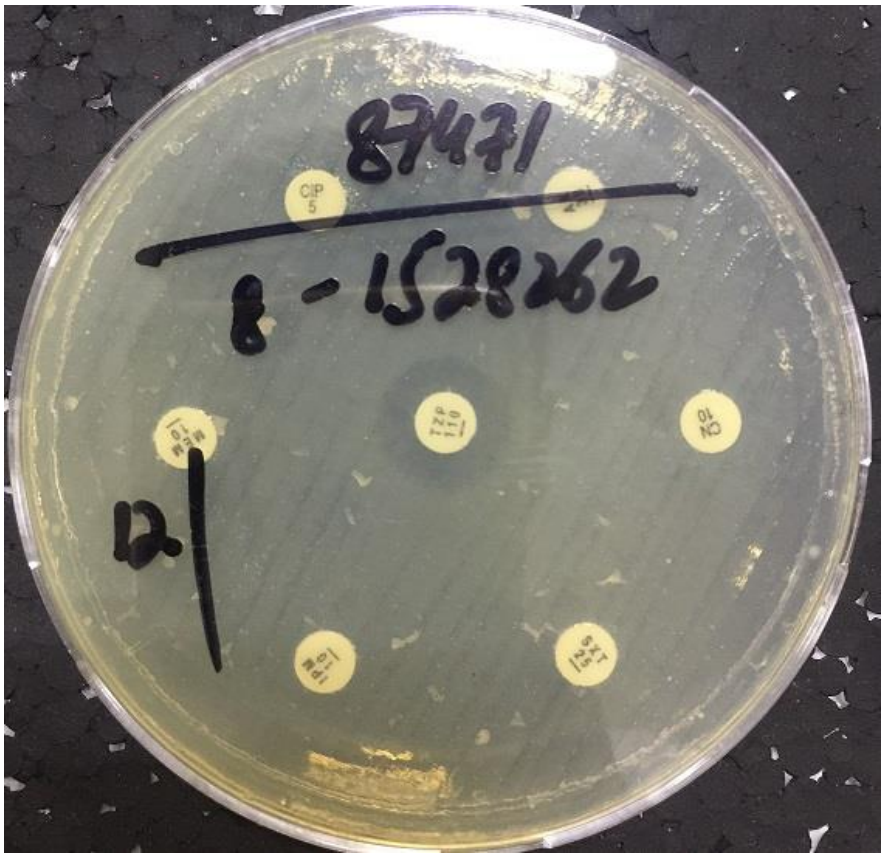

Fig: MEM and IMP and TZP Resistance

Table: III Percentage of Antibiotic Resistance

Antibiotic	Code	Resistant Sample	Sensitive Sample	Percentage of Resistance
Meropenem	MEM	34	16	68%

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Imipenem	IMP	46	4	92%
Polymyxin B	PB	32	18	64%
Gentamicin	CN	30	20	60%
Tobramycin Tazobactam	TOB TZP	8	42	16% 12%
		6	44	

***R=Resistant; S=Sensitive**

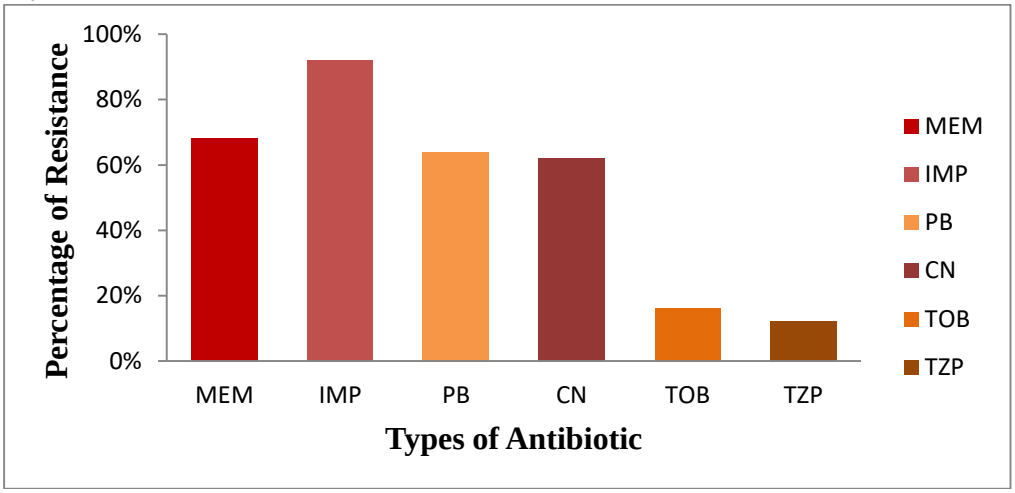

Figure: Antibiotic Resistance Graph

Detection of Carbapenem Resistant Gene

VIM gene is detected in *Pseudomonas aeruginosa* from cancer patient. It is detected from 42 samples as shown in Fig:

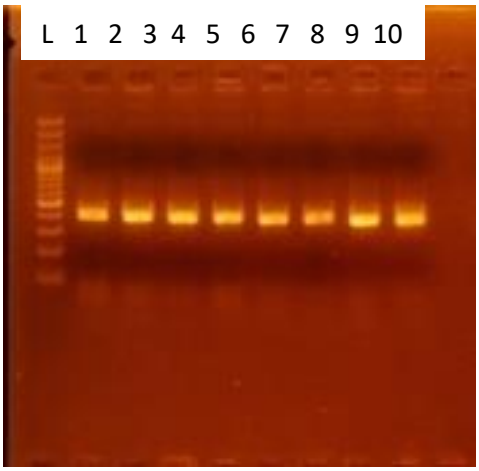

Figure: Detention of VIM genes

VIM gene is detected in 22 blood samples. Seven in urine samples and 13 in nasal samples as shown in Fig:

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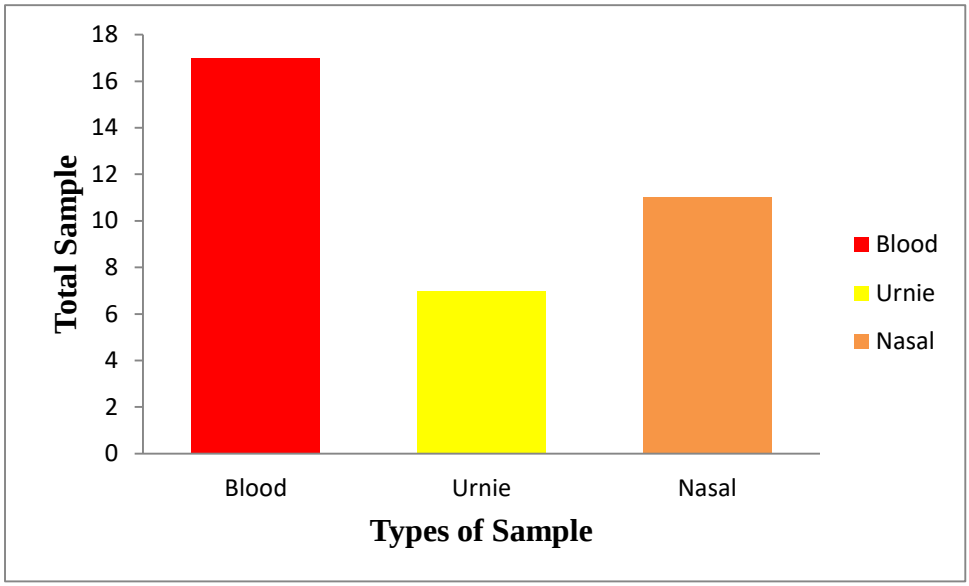
Figure:



Detection of Vim in different samples

Imp gene is detected from 36 samples in *Pseudomonas aeruginosa* from cancer patient as shown in Fig.

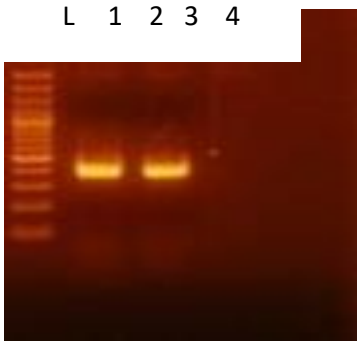


Figure: Detection of IMP genes

IMP gene is detected in 17 blood samples, 11 nasal samples and seven urine samples as shown in Fig:

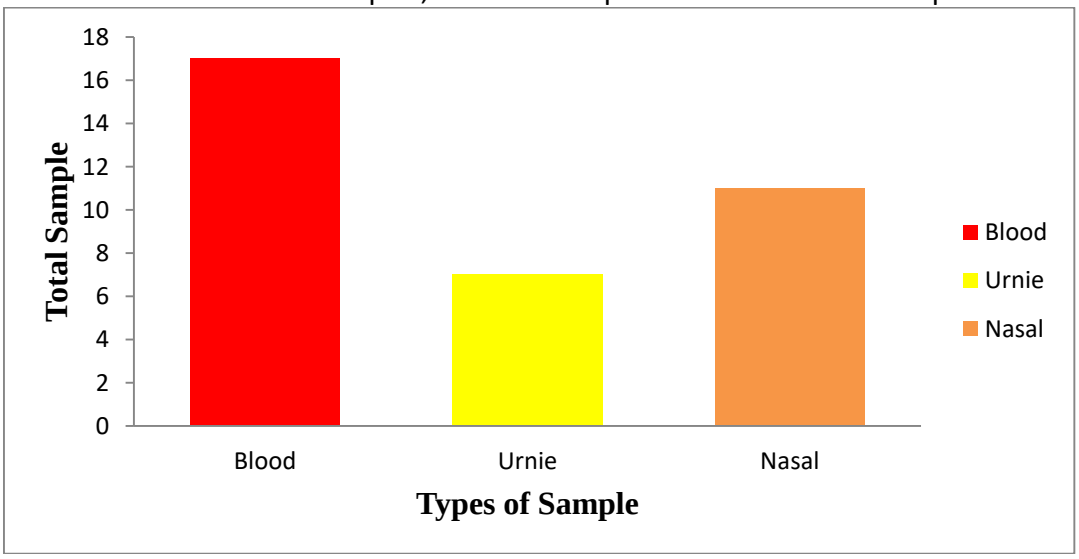


Figure: Detection of IMP from different sample

Discussion

The aim of this research was to isolate the multi-drug resistance *P. aeruginosa* and the detection of resistant gene from clinical samples of cancer patient. Fifty positive samples were obtained. The prevalence of *P. aeruginosa* in this study 33% showed that *P. aeruginosa* may potentially be significant in

causing infection in cancer patient. The highest prevalence of *P. aeruginosa* found in blood sample was 50% in comparison with urine sample which was 20% and the prevalence of nasal sample was 30% and the high prevalence relates to the results obtained in a study by (Picao *et al.,* 2009). Cancer is a problem in a medical field because due to the low immunity and compromised host defense microorganism has an idol condition to cause the disease. Multi drug resistance strains responsible for hospital acquired diseases, especially in the population at risk most commonly patient with cancer. MDR *P. aeruginosa* cause the major problems in cancer or cystic fibrosis patient (Khan *et al.,* 2015). The prevalence of *P. aeruginosa* in blood sample indicates that it poses a high risk in causing bacteremia in cancer patient. The intermediate prevalence of *P. aeruginosa* in nasal sample indicate that it may be transmitted through the physical content bacteria may be easily transmitted. The low prevalence in urine samples indicate that *P. aeruginosa* cause lesser urinary tract infection in cancer patient.*P. aeruginosa is* resistant to the majority of detergents and disinfectants (Toval *et al.,* 2015) the patient with low immunity and with compromised host defense at high risk of infection. The cross contamination with the hospital isolates plays the major role in causing nosocomial infections (Shah *et al.,* 2015). The elevated prevalence of *P. aeruginosa* 50% may be due to the multi drug resistance strains present in hospitals. The study reveals that the use of broad-spectrum antibiotics plays a most important role in the development of resistance against the antibiotics. There is a requirement to develop the antimicrobial and disinfectant which act on a biofilm formation and inhibit the biofilm formation (Rossoloini *et al.,* 2005). The prevalence of *Pseudomonas* in men was 34% in women was 46% and in child was 20%. A research conducted by (Souli *et al.,* 2008) reveals that the prevalence in males was higher than in females. The high prevalence in males could be due to males not being as particular as women in terms of hygiene. In this study *P. aeruginosa* shows antimicrobial drugs resistant against Meropenem (MEM) 68%, Imipenem (IMP) 92%, Polymyxin B (PB) 64%, Gentamicin (CN) 60%, and Tobramycin (TOB) 16%, Tazobactam (TZP) 12%

The best antibiotics which are used against the infection of multi drug resistant gram-negative bacteria are carbapenem. In current years, the countries which are seriously facing the problem of antibiotic resistance Egypt are one of them (Mahmoud *et al.,* 2013). In the present research resistance is high against all the commercially available antibiotics among *P. aeruginosa* isolated from the Shoukat Khanam Memorial Hospital and research center. The prevalence of resistance against Meropenem is 68% and the resistance against the Imipenem is 92%. The high rate of carbapenem resistance indicates the less treatment option in Hospital. This is due to the increase in antibiotic usage in the last past years due to this the bacteria modify the mechanism of resistance. In many other developing countries, the situation is same the resistance is on the increase (Picao *et al.,* 2009). Among gram negative bacteria the *P. aeruginosa* and Acinetobacter shows the high level of resistance against the Imipenem which is 37.03% the study was conducted by (Alipour *et al.,* 2010). Mahmoud *et al.,* 2013 conducted a research on a *P. aeruginosa* and reported that the 33.3% isolate resistant to Imipenem. In the countries which are located in a middle east Imipenem resistance is on the increase. In the Saudi Arabia the resistance against the Imipenem is 38.57% reported in 2011 (Mohmoud *et al.,* 2013). According to the European surveillance system in six different European countries the carbapenem resistance is reported about 25%. The highest resistance against the carbapenem reported in Greece which was 51% (Souli *et al.,* 2008). The Bacteria has different type of enzyme which plays an important role in the resistance.

The most commonly reported families are IMP which was firstly isolated in Japan. The VIM family was firstly isolated from Italy. SPM and AIM which was firstly isolated from Brazil. IMP and VIM producing *P. aeruginosa* are reported worldwide in different areas (Chadha *et al.,* 2014). In this current research VIM was the most commonly detectable gene among *P. aeruginosa.* The prevalence of VIM gene among *P. aeruginosa* was 82%. In the 72% isolates the IMP gene is detected. The result of previous studies supports the findings of our research. Previous studies demonstrate that the VIM is the most commonly prevalent gene among the *P. aeruginosa* and IMP gene prevalence also very high the greatest clinical

threat (Wolter *et al.,* 2009). In all over the world VIM gene is associated with the hospital outbreaks due to the MBL producing *P. aeruginosa* (Zhang *et al.,* 2013). In our study, 42 isolates are positive with the VIM gene and the 36 isolates are positive with IMP. These play a most significant role in multi-drug resistance.

Conclusions

The prevalence of beta lactamase gene producing isolates and their isolation from cancer patients is increasing in Pakistan, considerably Intensity pressure for usage of antimicrobial drugs by patients with Cancer, results in eradication of normal flora and consequent substitution of MDR strains. We have shown that genes and ß-lactamase producing *P. aeruginosa are* widespread in cancer and should be supervised by working on timely identification and strict isolation methods that will help to reduce the mortality and morbidity rate in these patients.

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