PREVALENCE OF CARBAPENEM RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED BY PHENOTYPIC AND GENOTYPIC (OXA-48) METHODS IN A TERTIARY CARE HOSPITAL

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DEDICATION

This thesis is dedicated to my lovely kids, beloved husband and respectable mother.

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LIST OF ABBREVIATION

S no	Abbreviations	Stand for
1	WHO	World Health Organization
2	OXA	Oxacillinase
3	VIM	Verona Integron encoded β lactamase
4	MHT	Modified Hodge Test
5	MCIM	Modified Carbapenem Inactivation Method
6	PCR	Polymerase chain reaction
7	CLSI	Clinical and Laboratory standards Institutes
8	CDC	Center for Disease and Control
9	VAP	Ventilator associated pneumonia
10	H2O2	Hydrogen per oxide
11	MDR	Multidrug resistance
12	XDR	Xtensive drug resistance
13	MGE	Mobile genetic elements
14	QSI	Qourum sensing inhibitors
15	CRE	Carbapenem resistant Enterobacteriaceae
16	IMP	Imipenemase
17	KPC	Klebsiella pneumonae carbapenemase
18	CRPA	Carbapenem resistant *Pseudomonas aeruginosa*
19	NDM	New Dehli Metallo β lactamase
20	ESBL	Extended spectrum β lactamase
21	MDRPA	Multidrug resistant Pseudomonas aeruginosa
22	CDDT	Combined Disc Diffusion Test
23	GIM	German Imipenemase
24	DIM	Dutch Imipenemase
25	AIM	Adelaide Imipenemase
26	SIM	Seoul Imipenemase
27	FIM	Florence Imipenemase
28	MH	Mueller Hinton agar
29	TSB	Triptic soya broth
30	AST	Antibiotic susceptibility test
31	CPO	Carbapenem producing organism
32	CCU	Cardiac Care Unit
33	PNS	Pakistan Naval Shifa

ABSTRACT

Pseudomonas aeruginosa is a human pathogen, it is a normal flora, but whenever opportunity arises it will cause disease. It is a very common pathogen involved in nosocomial infections. According to WHO (2017) infections caused by this organism have to face a challenge of resistance towards antibiotic carbapanem. Carbapenems are the drug of choice for serious infections and generally used for multi-drug resistant infections. These antibiotics are resistant to hydrolysis by enzyme β-lactamases which are produced by most of these microorganisms, nowadays their use as a last-choice treatment option has been compromised by the emergence of carbapenem inactivating enzymes called carbapenemases. These enzymes are produced by *Pseudomonas aeruginosa,* Acinetobacter bauminnii and Enterobacteriacea. Carbapenemases are classified according to Ambler molecular classes A, B and D coded by genes residing in plasmids, integron and transposon which often carry multiple resistance determinants limiting further treatment options. The aim of our study was to detect the presence of OXA-48 which has not been previously reported in our part of the world. We also tested by phenotypic methods carbapenemase resistance in isolates recovered in our center and then looked for the presence of OXA-48 genes in those isolates exhibiting resistance.

Objective

The intent of our study was to determine the prevalence of carbapenem resistance in isolates of *P. aeruginosa* among indoor patients of a tertiary care hospital Karachi. This resistance was tested by three phenotypic methods. We also did the molecular method for detection of resistance transcribing genes, although this resistance has been reported to be transcribed by a multitude of genes encoding for different forms of carbapenemases, we only detected OXA-48 gene which has not been previously reported in Pakistan.

Subject, Materials and Methods

This study was carried out at microbiology department in PNS Shifa Hospital from September 2018 to May 2019. This study consisted of 140 samples of *P. aeruginosa,*

which were received from different wards [ENT (ear, nose and throat), Plastic Surgery, Burn Unit, Medical wards, Pedriatics, Family ward and ICU (intensive care unit)] of PNS Shifa. These clinical samples were cultured on blood agar and Mac Conkey agar at 37^{o}C ±2^{o}C for 24-48 hours. Subsequently, we verified the cultured isolates biochemically by testing for oxidase production. When results were positive; antimicrobial disc (Meropenem and Imipenem 10µg) (oxoid) was applied through AST (antibiotic susceptibility test). When these carbapenem discs exhibited a resistance diameter of 15mm, then carbapenemase production was checked by both phenotypic methods like Modified Hodge test (MHT) and Modified Carbapenem Inactivation Test (mCIM). In case of carbapenem resistance, OXA-48 gene was detected by Real-Time PCR (Polymerase chain reaction).

Conclusion

Carbapanem resistance is an alarming threat all over the world in general and in particular in our geographical area; because of rampant misuse of antibiotics. We were successful to detect carbapenemase production phenotypically by MHT (Modified Hodge test) and mCIM (modified carbapenem inactivation method). These are screening methods for detection of carbapenemase. We found that mCIM is easy, affordable and accurate method for detection of carbapenemase. We are also first time reporting the presence of OXA-48 gene in isolates of *Pseudomonas aeruginosa* in Pakistan. We conclude from our findings that the implementations of screening tests are effective for the detection of resistant strains and should be done as a first stage of treatment strategy. This will prevent the evolution of gram negative organisms from multidrug resistant to pan-drug resistant because their genome is very dynamic and there is a continuous acquisition of resistance genes in bacterial populations. Infection control measures may be instituted in preventing the dissemination of these resistant bacteria.

Keywords: *Pseudomonas aeruginosa*, carbapanem resistance, OXA48 gene, CLSI, MHT, mCIM

CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous organism that can cause disease in all living beings including plants, animals and humans. It is a Gram negative, non-fastidious pathogen. A species of substantial medical significant *Pseudomonas aeruginosa* has the property of being a multidrug resistant organism known for its pervasive nature. It has inherent progressive antibiotic resistance and relationship with fatal disease. It is a common cause of hospital acquired infections like septic shock and ventilator associated pneumonia. This organism is recognized as an opportunistic one, as serious disease most frequently occurs in patients who are already have existing debilitating state most particularly cystic fibrosis and super-imposed infections in burn cases. It is a common pathogen in immune-compromised individuals but can also infect the immunocompetent people. It is found globally in soil, water and as a normal flora in man and most manmade environment (Buehrle DJ et al., 2017).

In all oligotropic environments which contain minimum of nutrition, *Pseudomonas aeruginosa* is the major inhabitant making it the top abundant organism in the world. Because it multiplies in damp clammy environment, these bacteria adhere to medical equipments like catheters causing cross infection in clinics and hospitals (Rosenthal et al., 2012, Zhao & Hu, 2015). Risk factors for these infections are intestinal colonization, previous use of antibiotics, fundamental disease and inappropriate antibiotic therapy. Treatment of *Pseudomonas aeruginosa* is difficult and more sophisticated antibiotics are required and adverse outcomes can occur (Meletis, 2016).

Every year 5000 hospitalized patients are estimated to die from persistent burn wound infections as a result of *Pseudomonas aeruginosa.* Sepsis as a result of resistant *Pseudomonas aeruginosa* is the foremost source of death in the United States, and the tenth major basis of death according to CDC (Centers for Disease and Control) (Greenwood, Slack & Barer 2012). Approximately 750,000 Americans are involved in sepsis, 30% out of which die every year. VAP (ventilator associated pneumonia) is also associated with *Pseudomonas aeruginosa* infections (Hajj J et al., 2018). According to statistics of India, ICU (intensive care unit) has an incidence of 30%-73% (Chaudhry and Prajapat 2017).

Nosocomial infections are considerably related with antibiotic-resistant organism especially in case of *Pseudomonas aeruginosa*. This resistance is acquired by several plasmid borne genes which encode enzymes that can degrade antibiotics or modify drugs rendering them in affective. Plasmid mediated resistance takes place at a higher rate than chromosomal mediated resistance (Mehrad B et al., 2015). GENEVA WHO has published the list of antibiotic resistant "priority pathogens" on 27th February 2017. This list emphasizes the hazards of Gram-negative bacteria that are related with resistance towards multidrugs. These bacteria are genetically equipped with resistance towards antibiotics. This list is a new tool to focus on health issues. In this list, *Pseudomonas aeruginosa* is included in priority 1 category that is resistant to carbapenem. Other microorganisms included in priority 2 and 3 category those are resistant to different antibiotics (Perales O, 2017).

There are several pathways of resistance to carbapenems including loss of porins channels, efflux pumps, diminished production of PBP and reduced affinity of PBP for carbapenemases and most significantly production of enzymes β-lactamase and OXA-carbapenemase. All these pathways are encoded by genes. According to the molecular level, the Beta-lactamases are classified into four types (A, B, C, and D). According to their amino-acid sequences, they are divided into two classes like amino acid serine β lactam (A, D) based on hydrolytic mechanism and Metallo –Beta-Lactamases (B) that contains Zinc in the active side (Hall & Barlow 2005). These are Group A (Kpc), Group B (IMP, VIM) and Group D (OXA) OXA23, OXA24/40, OXA48 and OXA58. Group (D) contains oxacillinases (OXAs), which are plasmid encoded and OXA 51 is chromosomally encoded (Zanganeh Z & Eftekhar F 2015).

This study highlights the progressing threat of antibiotic (carbapenem) resistance in *Pseudomonas aeruginosa* infections and the importance of antibiotic susceptibility testing to detect resistant strains. *Pseudomonas aeruginosa* isolates harbor resistance. There is an urgent need to institute infection control policies to prevent the dissemination of resistant strains.

1.1 **Nomenclature**

P. aeruginosa is a Greek word (pseudes) meaning "false unit".The word 'mon' was used as a germ in Microbiology (Kingdom Monera). *Aeruginosa* is a Latin word meaning verdigris "copper rust". But in Greek word "ae" meaning "aged" and "ruginosa" meaning "wrinkled".

It can produce different pigments like blue-green pigment (pyocyanin) as per Figure 1 and 2 on page 6, green (pyoverdin), red pigment (pyorubin) and black (pyomelanin). *P. aeruginosa* are Gram negative rod (pink) under microscope as per Figure 3 on page 7.

Basically "pyo" is a Greek word and its meaning "pus". It can produce pigments when grown on pigment enhancing media. Colonies are mucoid, big and flat with irregular and translucent borders. It has different virulence factors like exotoxin A, exoenzyme S, cytotoxic proteases, phospholipases, rhamnolipids, hydrogen cynide and toxic pigment pyocynin. *Pseudomonas aeruginosa* was found out in 1882 in Paris by a Chemist and Bacteriologist Carle Gessard, who identified this microbe by a blue green pigment when it is exposed to sun-light. It was recognized in the study "On the blue and green coloration of bandages" (Gessard C 1984).

1.2 Genome

The genome of *Pseudomonas aeruginosa* is great (5.5-6.8 Mbp) with 65% Guanine and Cytosine content. It is now obvious that *P. aeruginosa* consists of phenogenetically three different groups. Group 1 consists of PA01; Group 2 strains include the ST_{235} clonal linage and exo 4-positive. But Group 3 belongs to few strains like PA_7 (Bruggemann et al., 2018). It consists of accessory and core genome. The accessory genome belongs to genomic islands and islets from a primitive tRNA-integrated iseland type. The core genome shows a low level of nucleotide divergence. *P. aeruginosa* has a chromosome and plasmid. The plasmids, TEM, OXA, and PSE, encode for β-lactamase production. *P. aeruginosa* strains PA01 and *P. aeruginosa* PA14 have the complete genome sequence. The "*Pseudomonas* scientist" from the Washington PathoGenesis Corporation and the Department of Biology of the University of Calfornia, San Diego found the complete genome sequence of *Pseudomonas aeruginosa* PA01 in 2000 (Curran B., 2004).

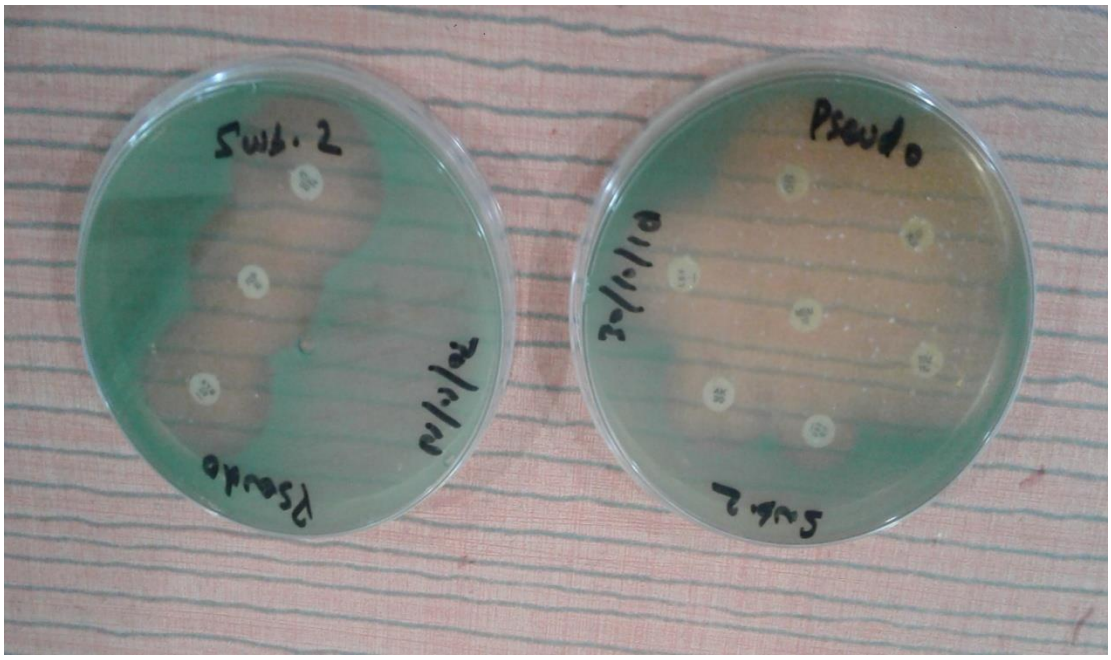


Figure1 (Green pigment Produced by *Pseudomonas aerugi*ue)

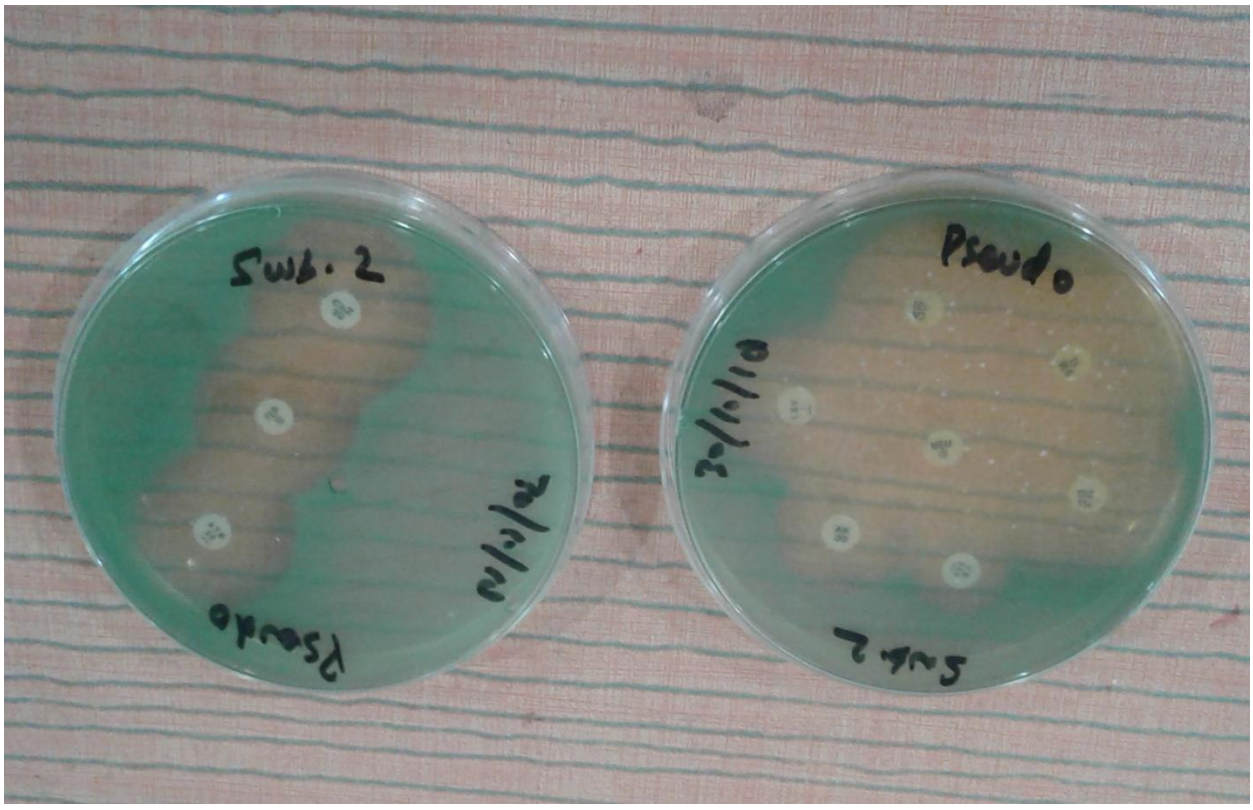


Figure 2 (Blue-green pigment produced by *Pseudomonas aeruginosa)*

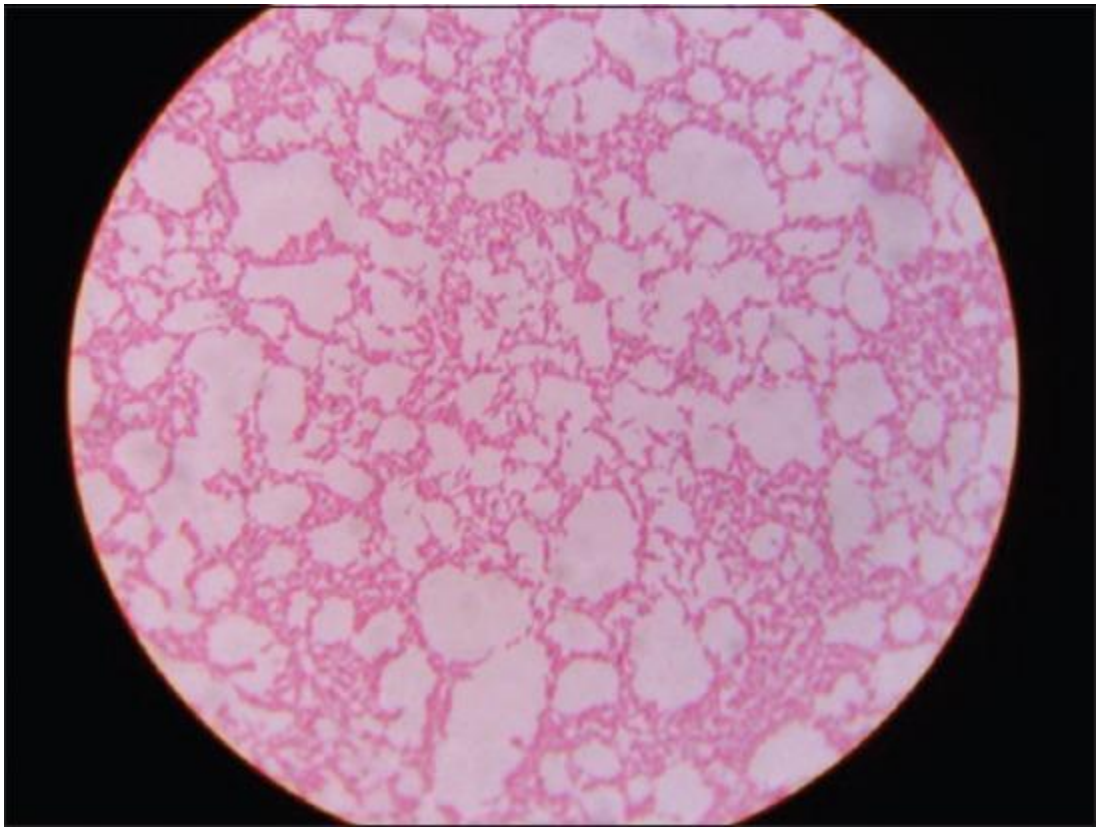


Figure 3 (Gram negative rod *P. aeruginosa*) (Banerjee et al., 2017)

This is the largest sequenced genome with inversion at rrnA and rrnB (Curran, Jonas & Grundmann, Dowson 2004).The *Pseudomonas aeruginosa* PA14 was sequenced in 2005 by Harvard Medical School scientists. It is different from *Pseudomonas aerugonosa* PA01 (as per Figure 4 on page 9) on the basis of insertion of the 107911bp in PA14, which was absent in PA01 (Stover et al., 2000).

1.3 Classification

Table 1 as per page 9

1.4 Metabolism

It is an aerobe but can grow anaerobically when nitrate is accessible. In absence of oxygen, nitrate and nitrite, it can ferment arginine and pyruvate by phosphorylation. In this way it gets 6-P-gluconate which will provide energy for cell. It can synthesize Fe^{-} or Mn^{-} containing superoxide dismutase enzymes. It breaks down the reactive oxygen to hydrogen per oxide and oxygen. *P. aeruginosa* consists of two siderophores like pyochelin and pyoverdin. It secretes these siderophores to the out of the cell and attaches to iron and takes it back to cell. In respiratory chain *P. aeruginosa* contains enzyme NQR (NADH ubiquinone oxidoreductase) which transports the electron from NADH to ubiquinone and facilitates the essential cellular processes (Raba et al., 2018).

P. aeruginosa consists of outer membrane OprF. It is a structural protein and sustains cell shape. It acts as a porin and reduces the intake of injurious things into cell with limit of 500Da. It consists of flagellum and pili. Flagellum helps in movement and chemotaxis for sugars. It has two types of flagella, strains a-type and b-type. It spreads infection by attachment of flagella with host cell. It can adhere to mucosal and epithelial surface with help of pili. Both flagellum and pili are moved with help of RpoN (Alhazmi A, 2015).

1.5 Ecology

It is a versatile organism on the basis of different enzymes as it can utilize different substances as nutrients. It can grow in mucoid environment by having alginate.

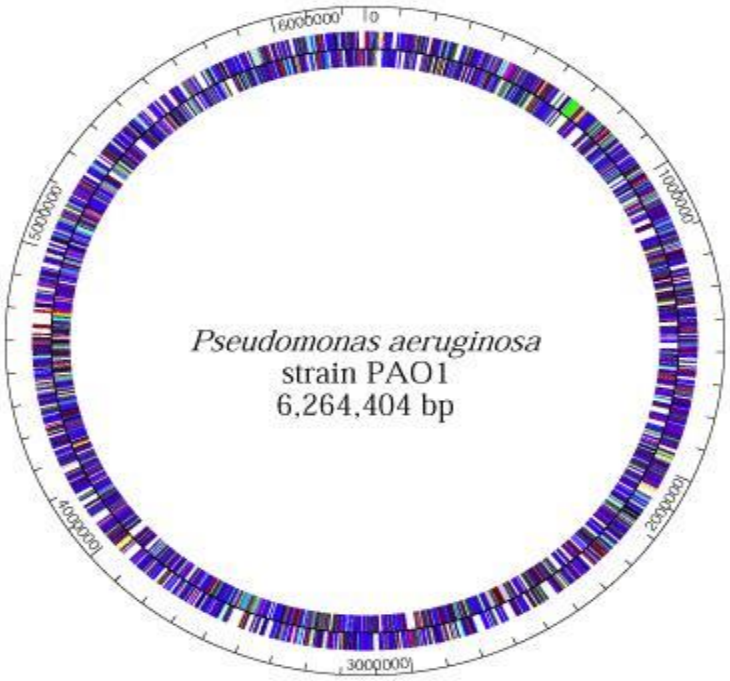


Figure 4 (*P. aeruginosa* strain PAO1) (Stover, C. K et al., 2000)

Table 1

Classification of *P. aeruginosa*

Domain	**Bacteria**
Phylum	**Proteobacteria**
Class	**Gamma proteobacteria**
Order	**Pseudomonadales**
Family	**Pseudomonadaceae**
Genus	***Pseudomonas***
Species Group	***Pseudomonas aeruginosa* group**

Ref (Lederberg, Joshua et al., 2000)

Basically *P. aeruginosa* can support the growth of plants by degrading polycyclic aromatic hydrocarbon. But some strains can infect plants also. *P. aeruginosa* can form biofilms with the help of that it can stick to the different surfaces like metals, plastics, tissues and implants. There are 5 stages of biofilm formation. In stage I, it adheres to surface (as per Figure 5 on page 11). In stage II, it will be irreversible. In stage III microcolony is formed. Stage IV consists of biofilm maturation. In stage V dispersion occurs (Rasamiravaka, Labtani, Duez & El Jaziri 2015).

Biofilm formation is a serious problem for medical care in immunocompromised patients and elderly. Traditional antibiotics are not effective against them (Moradali MF et al., 2017).

1.6 Pathogenesis

P. aeruginosa hardly causes infection in healthy people but colonization with *P. aeruginosa* leads to hospital acquired infections but exact mechanisms of transmission are unknown as it is omnipresent in the environment. Previously rectal colonization was associated with infections but Fluoroquinolones and Carbapenem utility selected for digestive tract colonization with MDR/XDR (multidrug resistant/extensive drug resistant) strains. In colonized patients chances of developing *Pseudomonal* infections are 15 times more than non colonized patients (Rice 2018; Murray, Kwon, Marcotte & Whiteley 2015).

It is an ubiquitous and sturdy organism that can grow in distill water. There is an adaptation of German proverb "In wine there is truth; in beer there is strength, in water there are *Pseudomonads*" (Greenwood D et al., 2012).

Pseudomonal infections are common in unconscious patients, craniocerebral trauma, drainage tubes in any part of body and tracheostomy. *Pseudomonas* has different species like *Pseudomonas aeruginosa, Burkholderia pseudomallei* and *Burkholderia cepacia complex* which are responsible for different human infections. Mucoid forms of *P. aeruginosa* are responsible for cystic fibrosis. It is an autosomal recessive disorder accompanied by mutation on chromosome 7. As a result of chloride channel defaults salt and water balance is deranged and leads to accumulation of thick mucus.

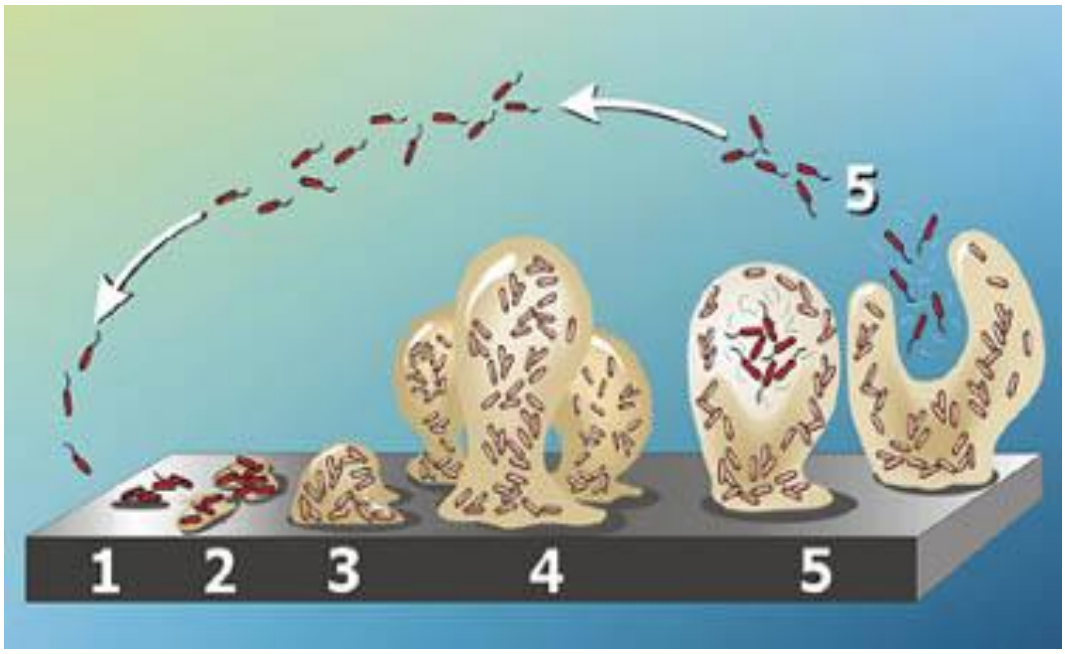


Figure 5 Stages of Biofilm formation (Moskowitz SM et al., 2004)

Figure 6 Mucoid colonies of *Pseudomonas aeruginosa* (microbewiki.kenyon.edu)

This thick mucus (as per Figure 6 on page 11) can facilitate the growth of *P. aeruginosa.* It is also a major organism in patients who are on corticosteroid therapy, radiotherapy and chemotherapy (Bruggemann et al., 2018).

According to center of disease control (CDC), it is a widespread cause of hospital acquired infections (Centre for disease control and prevention 2012).

The CDC deals with surveillance, prevention and help to minimize the drug resistance towards serious infections. Bacteria have survived in this world for three billion years and have acclimatized themselves against threats for survival. *Pseudomonas aeruginosa* even has been isolated from dust clouds. Antibiotics have been used against microbial infections for more than six decades. Antibiotics have transformed medical care in the 20^{th} century, but now-a-days microorganisms have been succeeding the fight against the therapy options by adapting different survival strategies (Aslam B et al., 2018).

Virulence factors

Pseudomonal infections can be spread to the burn patients through fomites, vectors and hospital workers. Otherwise those bugs already present on skin get entry into bloodstream. Pili, flagella, and enzymes like elastase and proteases also play an important role in spread of burn infections (Gonzalez M et al., 2016).

- **Lipopolysaccharides (LPS)** play an important role in its pathogenesis.

  a. Lipid A

  It is known as endotoxin. It initiates inflammatory reaction by interacting with host receptors (Alazmi A, 2015).

  b. O antigen-side chain

  It is resistant to human serum and antibiotics (Alazmi A, 2015).

- **Polar flagella**

  It facilitates motility and activates IL-8 by interacting with toll like receptors in airway epithelial cells. It promotes adhesion (Alazmi A, 2015).

- **Exotoxin A**

  It leads to direct tissue damage and necrosis (Alazmi A, 2015).

- **Type III secretory system**

  It facilitates the injection of virulence factor into cell. Leukocidin can damage neutrophils (Alazmi A, 2015).

- **Alginate**

  It serves to hide the *Pseudomonas aeruginosa* from the immune system (Alazmi A, 2015).

- **Quorum-sensing (QS**)

  It permits *Pseudomonas aeruginosa* to react to their population mass by expressing genes. In case of high population mass, two toxins (elastase and pyocyanin) of *Pseudomonas aeruginosa* are produced as a result of quorum sensing signaling. Quorum-sensing plays an important role in communications with other cells with the help of enzyme tyrosine phosphatase. This will facilitate the biofilm synthesis (Alazmi A, 2015).

- **Biofilm formation**

  Biofilm is a thin layer, in which communities of *Pseudomonas aeruginosa* are located in a matrix of extracellular polymeric substance that communicates them to the external surface. The process of biofilm synthesis starts with reversible adhesion, followed by irreversible adhesion with the formation of micro colonies of *Pseudomonas*. These colonies grow bigger with passage of time and meet together to form non colonized space. This space is filled with bacteria and covers the whole surface (Alazmi A, 2015).

- **Immune response**

Pseudomonas deteriorates the human's first line of defense especially in burn cases. *Pseudomonas aeruginosa* is liable to opsonic phagocytosis. This type of phagocytosis is determined by antibody-binding of Fc-receptors and complement receptor. In case of burn infections, Fc receptor expression is diminished and leads to condense chemotaxis and polymorphonuclear cell fails to respond. In burn cases myeloid-derived suppressor cells lead to increased amount of arginase. This can reduce the L-arginine (L-Arg). As a result process of inflammation is increased. Less quantity of arginine causes T-cell being arrested in the G_0-G_1 phase of the cell cycle so activation and proliferation of the immune

system is prohibited. In burn cases, revascularization of wound blocks neutrophils motility towards wound infection (Everett et al., 2017).

1.7 Toxins

P. aeruginosa consists of different toxins (as per Figure 7 on page 15) like exotoxin A, exoenzyme S, cytotoxic proteases, phospholipases, rhamnolipid, hydrogen cyanide and pyocyanin. Exotoxin A can block eukaryotic elongation factor 2 through ADP-ribosylation, and without elongation factor 2 eukaryotic cell can necrotize. It also contains exo-enzyme Exo U which breaks down the membrane and responsible for cell lysis. *Pseudomonas aeruginosa* can show sensory system like quorum sensing that regulates the expression of virulence factors. The type III secretion system, that is accountable for inoculation of toxin in the cytoplasm of host cells. Proteases like elastases and exotoxins are associated with burn infections and septicemia (Alhazmi, 2018).

1.8 Detection and identification

- **Macromorphology** Big, flat and greenish blue colonies (2-4mm in diameter) are with rough borders. A clear zone of hemolysis is visible on blood agar sometimes. It has particular smell of caramel and strawberry soda. It can produce pigments like green fluorescent pigment, pyoverdine and blue pigment pyocyanin (O' Hara NB et al., 2017).
- **Micro morphology:** This organism appears as small pink rods.
- **Gram staining** It appears as Gram negative pink rods.
- **MacConkey agar** It gives yellow color because it cannot ferment lactose.
- **Biochemical test as per Table 2 on page 16**
- **Role of antibiotics for *Pseudomonal* infection**

The antibiotics with susceptibility action towards *Pseudomonas aeruginosa* are:

- Aminoglycosides **(**gentamicin, amikacin, tobramycin, but not kenamycin)
- Quinolones (ciprofloxacin, levofloxacin, but not moxifloxacin)

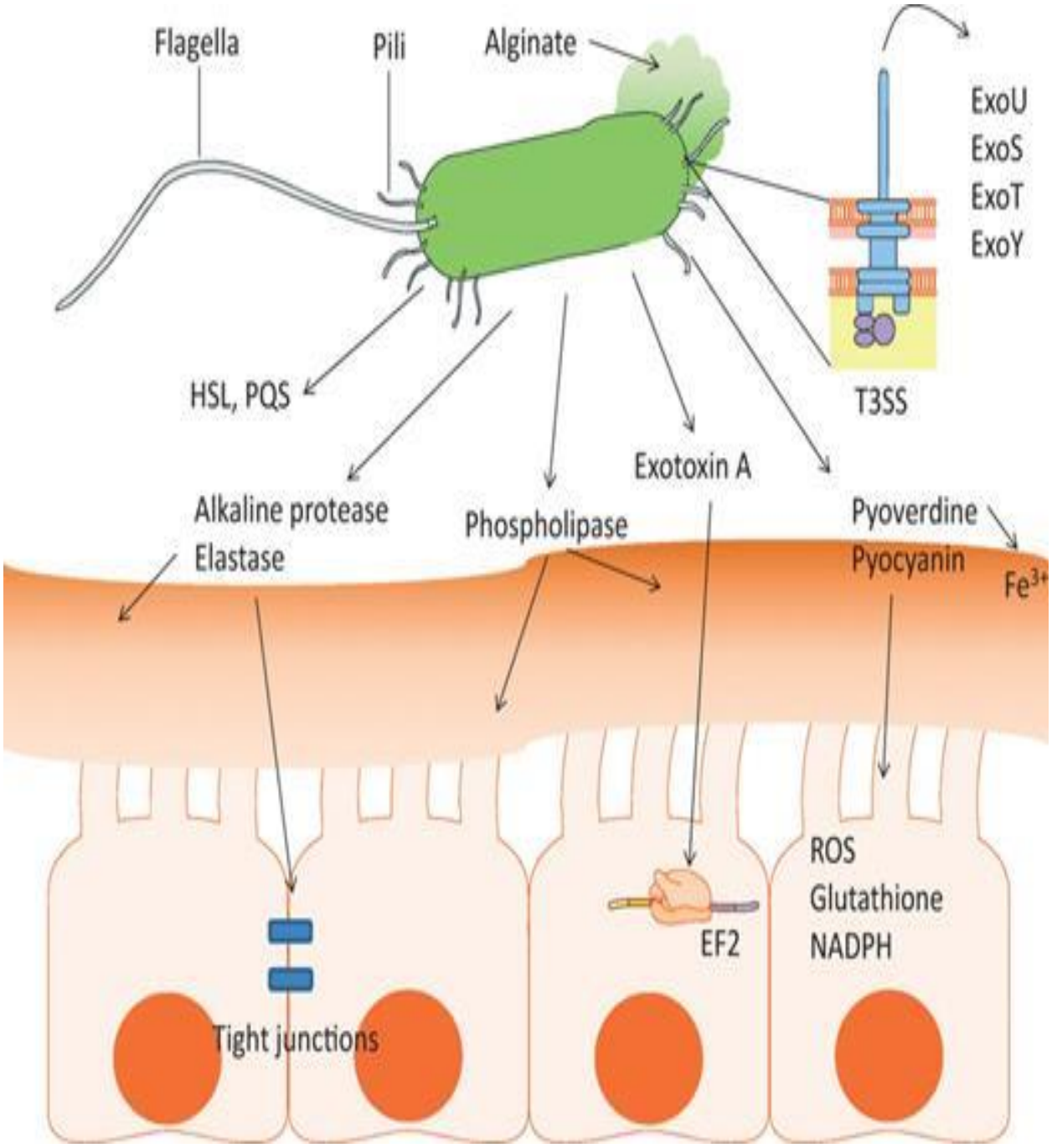


Figure 7 Toxins of *Pseudomonas aeruginosa* Gellatly SL & Hancock. (2013)

Table 2

Biochemical tests for detection of *Pseudomonas aeruginosa*

TEST	**RESULT**
Oxidase	Positive
Indole production	Negative
Methyl Red	Negative
Voges-proskaeur	Negative
Citrate	Positive
Hydrogen sulfide production	Negative
Urea hydrolysis	Negative
Catalase	Positive

Reference Sagar Aryal. (2018)

- Cephalosporins (ceftazidime, cefepime, cefoperazone, cefpirome, ceftobiprole, but not cefuroxime, cefotaxime, or ceftriaxone)
- Anti*pseudomon*al pencillins (carboxypencillins and ureidopencillins).
- Carbapenems (meropenems, imipenem, doripenem, but not ertapenem)
- Polymyxins (polymyxin B and colistin)
- Monobactams (aztreonam) (Bassetti M et al., 2018).

1.9 Carbapenem antimicrobial drugs

Carbapenem is the 4:5 fused rings of penicillins. It consists of double bond C-2 and C-3, but sulphur is replased by carbon at C-1. It acquires the hydroxyethyl side chain of thienamycin and plays an important role in its activity. Although thienamycin explains persuasive antibacterial and β-lactamase inhibiting effects. Thienamycin is a "natural product" and plays an important role in discovery of Imipenem (Papp-Wallace & Bonomo, 2016).

Thienamycin attached to PBPs (penicillin binding proteins) and blocked microbial activity against Gram-negative micro-organisms especially *Pseudomonas aeruginosa* and anaerobes (Bacteroid fragilis). Unluckily thienamycin was unstable in aqueous solution, receptive to hydroxylamine and cysteine (Papp-Wallace, Endimiani, Taracila & Bonomo, 2011).

The N-formimidoyl derivatives, imipenem were firstly discovered. Imipenem was the first carbapenem available drug. Unfortunately imipenem was destroyed by DHP-1 (Dehydropeptidase) in kidney, so it was co-administered with cilastatin and betamipron. In succeeding two decades, different modifications were done by adding 1-β-methyl and a pyrrolidine ring at C-2 to provide protection against DHP-1 hydrolysis. Different compounds like meropenem, doripenem and ertapenem were discovered. Meropenem was discovered in 1995 and it was consider as "Golden Ages" of antibiotics (Gould, 2016).

Chemistry: C-1 and transconfiguration of β-lactam ring at C-5 and C-6 plays an important role in stability against β-lactamases. The hydroxyethyl R_2 side chain provides resistance to hydrolysis by β-lactamases (Papp-Wallace & Bonomo, 2016).

Synthesis: Starting material, like natural products (L-Cys, L-Val, L-alpha-amino adipic acid, and S-adenosyl-Met), was utilized for manufacturing carbapenem. When carbapenem was produced which consists of R configuration at C-8, methyl at C-1, trans at C5-C6 and hydroxyl-ethyl at C-6, R1 side chain at C-2. It occupied two side chains R1 and R2, so it was different from other β-lactam antibiotics (Papp-Wallace & Bonomo, 2016).

Mode of action: For carbapenem antibiotic, it inhibits cell wall synthesis of *P. aeruginosa.* It penetrates through cell wall and then binds with PBPs (penicillin binding proteins) (Papp-Wallace & Bonomo, 2016).

Antimicrobial activity: Carbapenems are affective against both gram positive and gram negative microorganisms. Ertapenem is less effective than meropenem and imipenem against *P. aeruginosa*. MICs (minimum inhibitory concentration) of doripenem are lower than meropenem and imipenem against *P. aeruginosa*. In case of multidrug resistance carbapenems are used in combination with other drugs in order to control serious infections (Papp-Wallace & Bonomo, 2016).

Pharmacokinetics: Oral bioavailability of carbapenem is low, they cannot cross gastrointestinal membrane. Meropenem is given intravenously and imipenem by intramuscular injections. Carbapenems are excreted by renal route (Papp-Wallace & Bonomo, 2016).

Adverse effects: It can cause nephrotoxicity and neurotoxicity (Papp-Wallace & Bonomo, 2016).

Role of antibiotic resistance: Penicillin was the first antibiotic discovered by Alexander Fleming in 1929, but it was not until the early 1940s that its true potential was acknowledged and large scale fermentation processes were developed for the production of antibiotics. In the early years, new antibiotics were developed resistance against microorganisms, but recently antibiotic resistance towards pathogens is increasing all over the world. Antibiotic resistance is sneakily move closer and increasing among hospitalized patients. It is difficult to estimate how much antibiotic resistance will rise up in future. If we are not able to decrease the level of resistance against infectious diseases

due to microorganisms can take more life of human beings per year as compare to malignancies by 2050 (Schwartz T et al., 2015).

WHO has published the list of priority resistant pathogens on 27th February 2017. This list emphasizes the hazards of Gram-negative bacteria that are related with resistance towards multiple drugs. These bacteria are genetically equipped with resistance towards antibiotics. This list focuses on health issues coating *Pseudomonas aeruginosa* as priority 1 category that is resistant to carbapenem. Priority 1 is critical level and consists of *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacteriacea* (Morrow et al. 2013). This is the most critical group that presents danger in hospital, nursing homes and patients who are on ventilators and blood catheters. They are a great risk of blood stream infections and pneumonia. Priority 2 is high level and includes *Enterococcus faecium, Staphylococcuc aereus, Helicobacter pylori, Campylobacter, Salmonellae and Neisseria gonorrhoeae.* These organisms are resistant to multiple antibiotics like vancomycin, methicillin, clarithromycin, cephalosporin and fluoroquinolone. Priority 3 is medium level and consists of *Streptococcus pneumoniae, Haemophilus influenzae and Shigella.* These organisms are resistant to penicillin, ampicillin and fluoroquinolone (Perales, 2017).

Mechanism of resistance: Mechanisms of resistance to carbapenems (as per Figure 8 on page 20) consist of β-lactamases, efflux pumps, mutation of porins and penicillin binding protein. *Pseudomonas aeruginosa* strain exhibits both intrinsic and acquired resistance to carbapenem antibiotics. *Pseudomonas aeruginosa* genome is related with significant number of enzymes, efflux pumps that are responsible for intrinsic resistance towards carbapenems. Resistance is supplimentarily attained by mutation or horizontal gene transfer, so *Pseudomonas aeruginosa* has increased capability of getting multidrug resistance (Golle, Janezic & Rupnik, 2017). Intrinsic resistance of *Pseudomonas*

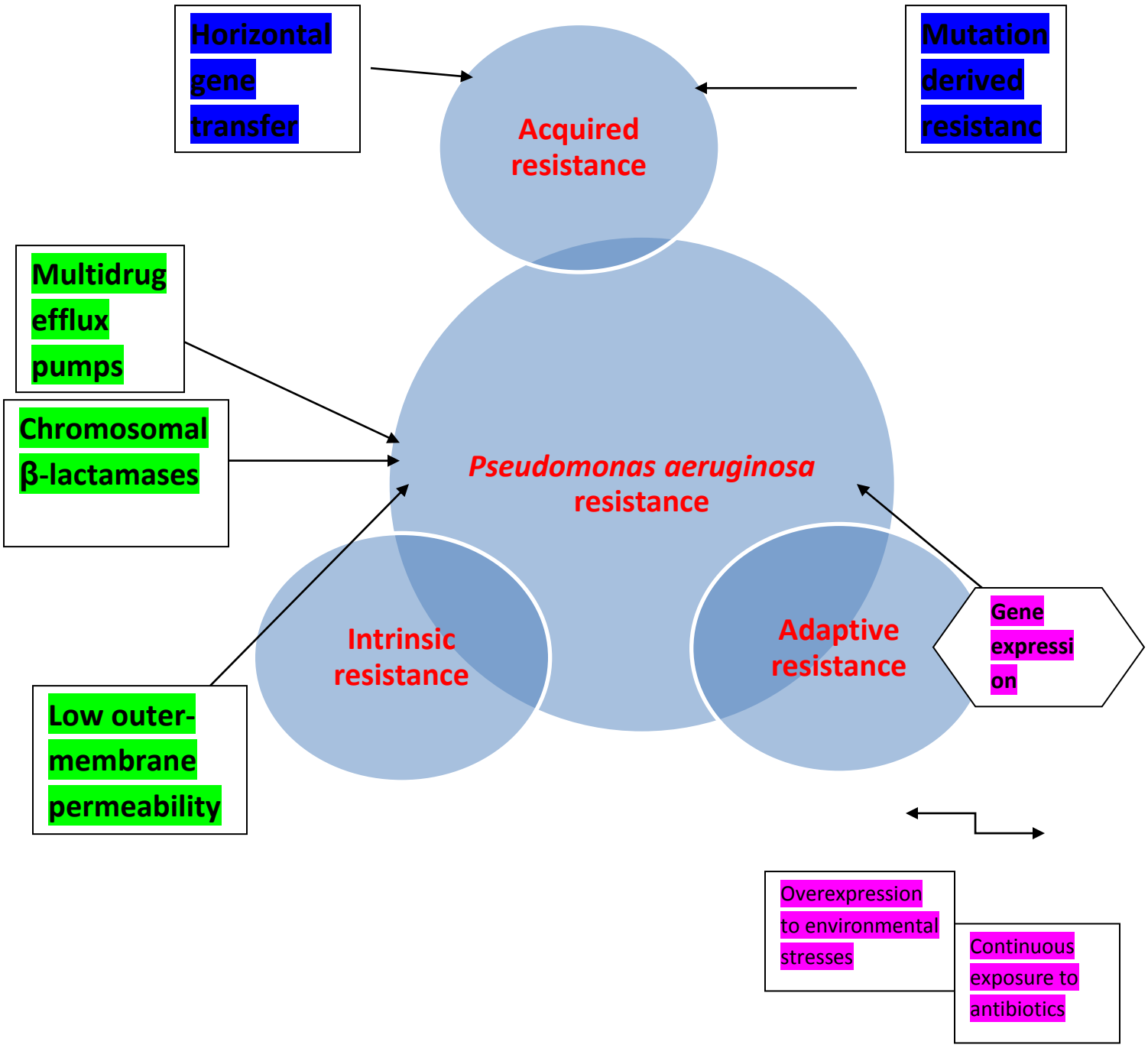


Figure 8 Mechanism of antibiotic (carbapenem) Resistance (Chatterjee M., 2015)

aeruginosa depends on impermeability. This impermeability is mediated by Mex A-Mex B-OprM. Mex B protein is siuated in the cytoplasmic membrane, the OprM protein is a pore-forming protein; and the MexA protein connects these components. Upregulation of Mex A-MexB-OprM is related with nalB mutation, and increases the MICs of penicillins, cephalosporins, quinolones, tetracycline and chloramphenicol but imipenem is excluded, because this carbapenem deficients lipophilic phenyl or heterocyclic side chain. Basically intrinsic resistance has limited the drug selection and also increases the peril for development of acquired resistance. In hospital *Pseudomonal* infections can be transmitted by nurses, instruments and food. These infections are hazardious in hospitalized patients because firstly it can lead to death as a result of pneumonia and secondly as these exhibit resistance towards multidrugs (Yayan, Ghebremdhin & Rasche, 2015; Cabot et al., 2016).

The genome of *Pseudomonas aeruginosa* is remarkably large (6.3Mb). Plasmid is extrachromosomal part of DNA that doesn't have genetic information. It consists of different types; one of them is R plasmid ("resistance" factors) that exhibits resistance towards antibiotics. Broad- host-range Inc (incompability) P-1, an extra-chromosomal genetic material, can transmit and reproduce in all Gram-negative bacteria. Inc-P plasmids are responsible for extending genes between dissimilar micro-organisms. These genes are related with resistance towards antibiotics. Antibiotic resistance expands through multifaceted communications by attainment of mobile genes that have advanced over time in bacteria of the environment. The pool of resistance genes in the environment is because of combination of naturally occurring resistance in human and animals waste, pollutants and microorganisms which cluches multiple resistance genes. The most considerable pool of multidrug resistant Gram negative bacilli is in the intestine of human beings and animals, predominantly those who are on use of antimicrobial drugs. Whenever water, food and the environment are infected with multidrug resistance Gram negative microorganisms will become a great source of resistance. The mobilome are the mobile genes that carry resistance towards antibiotics, and can be transmitted between faintly related bacteria of different phyla. Antibiotics are excreted by human beings in sewerage system. Now amalgam is discharged to rivers. It will approach agricultural systems whenever this slush is utilized as manure. Some case will happen to veterinary

antibiotics. Finally it can be transferred to ground water. So this is an ecological condition for horizontal gene transfer. Environmental studies have illustrated the allocation of Inc-P like replicons in fertilizers, soils and wastewater. RK_2 was the first discovered in out breaks of antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella aerugenes* in Birmingham in 1969. Plasmid in the Inc P-1 was detected from wastewater, soils and hospitals (Popowaska & Kraeczyk-Balska 2013).

Whenever plasmid is more than 20kb, it can endorse the transfer of genes through conjugation. In bacteria heritable genetics disparity occurs through mutation, this diffident evolutionary shifts exhibit antibiotic resistance (Bush K, 2018).

Scientists supposed that genes were fixed constituent of chromosomes till 1950. Barbara McClintock illustrated in 1951 that genes can shift from one chromosome to another one. These are known as transposons. These are responsible for antibiotic resistance. *Pseudomonas aeruginosa* acquires high intrinsic resistance. It has lately been categorized as an ESKAPE (Enterococcus *faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species). These microorganisms exhibit resistance towards antibiotics in hospitalized patients. It is related with intrinsic resistance (Gomez-Zorrilla et al., 2014; Juan, Pena & Oliver, 2017).

Carbapenems have extensive range of action against Gram positive and Gram-negative microorganisms. Carbapenems are bactericidal beta-lactam antibiotics like penicillins but sulphur atom at position 1 is replaced by carbon atom. This drug hampers the synthesis of the pathogen cell wall by binding and blocking the activity of penicillin–binding proteins. It has good efficiency against Gram-negative bacteria like *Enterobacter, E coli, Morganella morganii* and *Klebsiella*. For *P. aeruginosa* doripenem and meropenem are very effective but imipenem has slightly less activity. On the other hand ertapenem is not effective against *P. aeruginosa*. Carbapenems are used in case of abdominal infections but not as first line therapy. Similarly they are not working against atypical bacteria because these bacteria did not have cell wall that carbapenems attack (Shields et al. 2016).

Carbapenems are trustworthy drugs for bacterial infections but reported as their resistance is alarming. These drugs are broad spectrum and act against both Gram positive and Gram negative microorganisms (Marsik & Nambiar, 2011).

They act by different mechanisms like loss of porins, over-expression of efflux pump, β-lactamases and so on. According to the molecular level, the β-lactamases are classified into four types (A, B, C and D). This is known as Ambler classification. According to their amino acid sequences and inhibitor profile, they are divided into four sub classes(A, C, D) and fourth one is (B) that contains zinc at active side (Hall & Barlow, 2005). Among these classes C is insignificant. They have different enzymes like Group A carbapenemases (Kpc and GES enzymes), Group B metallo-β lactamases (IMP, VIM and NDM β-lactamases) and Group D carbapenemases (OXA23,24/40, 48, 51, 55, 143) have recently emerged (187, 189, 242, 243, 253). OXA carbapenamase is a great jeopardy of carbapenem resistance (Mohanty, Maurya, Gaind & Deb, 2013).

Group D contains oxacillinases (OXA), having OXA-1, OXA-2, OXA-48 and OXA-58 families (plasmid encoded) and OXA-51 (chromosomally encoded). Sykes and Mathew identified three distinctive oxacillinases like OXA-1, OXA-2 and OXA-3.The first plasmid gene was sequenced RGN_{238} (Sugumar et al., 2014).

OXA-48 type carbapenemases have been found in Turkey, North African Countries, the Middle East and India. OXA-48 was first detected in *Klebsiella pneumoniae* that was found in Istanbul, Turkey, in 2001. OXA-48 is evocative of OXA-10 which exhibits slight active site differences. The β5-β6 loop of OXA-24/40 and OXA-48 has same orientation and sizes which depend on carbapenem turnover, but substrate specificity of both enzymes is variable. The OXA-β-lactam with carbapenemases activity was primarily illustrated by (Paton et al., 1993).

Carbapenemases are group of enzymes that are able to break down carbapenems including imipenem, meropenem and other carbapenem drugs. Meropenem was first clinically applied in United States of America in 1996 (Rhomberg, 2006). Meropenem is bactericidal drug; it blocks the production of cell-wall of microorganism. It cannot be deprivated by enzymes like β-lactamases. It is stable to dehydropeptidases-1 inactivation,

more effective against Gram negative bacteria especially *Pseudomonas aeriginosa* (Rhomberg et al., 2006). Imipenem was first exercised clinically in United States of America in 1985 (Barnbuam J., 1985).

In case of *Pseudomonas aeruginosa* resistance can occur through loss of porin (OprD). Decreased expression of this pump results in diminished entrance of carbapenem into periplasm of *P. aeruginosa.* Expression is reduced as a result of mutation of oprD gene. The physiological role of this pump is the movement of amino-acids. It is specific pump that facilitates the entery of carbapenem like imipenem into *P. aeruginosa.*Over-expression of efflux pump is also responsible for carbapenem resistance. Two efflux-pumps like OprM and OprJ exhibit resistance towards carbapenem in case of *P. aeroginosa.* Basically efflux pumps are responsible for elimination of carbapenems. Cytoplasm is connected to outside of the cell by efflux pumps. This pump has three components like cytoplasmic membrane pump, a cytoplasmic membrane linker and an outer membrane-periplasmic channel/efflux porins. Mutation of penicillin binding protein constitutes the carbapenem resistance in *P. aeruginosa* and *Acinetobacter bauminii.* Low molecular weight penicillin binding proteins possess β-lactamase activity and antibiotic resistance. Genome of *P. aeruginosa* is 10% flexible. This flexible genome consists of chromosome, integrons and plasmids and known as mobile genetic elements (MGEs). Mobile genetic elements play an important role in antibiotic-resistance (Lambert PA, 2002).

1.10 EPIDEMIOLOGY

As per Table 3 (a), 3(b) on page 26 and 4 on page 27

1.11 Potential of alternative antimicrobial substances against *Pseudomonas aeruginosa* isolates

Garlic (Allium sativum) plays an important role to enhance the susceptibility of *P. aeruginosa* biofilm towards antimicrobial drugs. Azithromycin also plays the same role as QSI (Quorum sensing inhibitors). Bacteriophages are viruses that damage bacterial cell. They are naturally-occurring in the environment and they have capability to

penetrate the biofilm leads to bacterial death. Bacteriophage therapy has been utilized in Georgia (Hurley, Camara & Smyth, 2012).

Honey is affective in healing wounds specially burn wounds. It blocks the growth of bacteria especially *P. aeruginosa* as a result of high osmolarity. It is more effective than silver sulfadiazine (Henriques AF, 2011).

1.12 Role of antiseptics and disinfectants

Nosocomial infections present a grave danger of enhancing the morbidity and mortality. The rate of nosocomial infections is high in poor country as Pakistan. Water is a great source of *P. aeruginosa* in health care facilities. *Pseudomonal* infection can be transferred from contaminated sinks. It is hard to eradicate it completely but constant cleaning with chlorine or related disinfectant will stop the contamination (Dancer, 2014).

Table 3 (a)

Prevalent Carbapenemase genes in Pakistan

Carbapenemase producing genes	Number
BlaNDM	**31**
blaOXA-181/232	**15**
BlaVIM	**8**
blaOXA-23	**8**
blaOXA-51	**8**
BlaGES	**3**
BlaIMP	**1**
blaSIM-1	**1**

Reference (Braun SD et al 2018)

Table 3 (b)

Carbapenemase producing organisms with antimicrobial resistance genes in Pakistan

S. No	Carbapenemase producing pathogens	Antimicrobial resistant genes
1.	**Ecoli**	**blaNDM, blaOXA-2, blaIMP, blaKPC**
2.	**Klebsiella Oxytoca**	**blaVIM, blaNDM**
3.	**Klebsiella Pneumoniae**	**blaKPC, blaIMP, blaOXA181/232, blaOXA-1, blaSHV**
4.	**Proteus mirabilis**	**blaNDM, blaOXA-10**
5.	**Pseudomonas aeruginosa**	**BlaVIM, blaIMP, blaGES, blaSPM-1, blaOXA-18**

Reference (Braun SD et al 2018)

Table 4

Prevalence of Carbapenemase (OXA-48) in neighbouring countries

Year	Country	Carbapenemase Gene	Prevalence rate	References
2017	India	OXA-48	24.7%	Mohanty S, 2017
2018	India	OXA-48	28.2%	Kazi M, 2018
2016	China	OXA-48	62.2%	Guo L, 2016
2018	Iran	OXA-48	90.2%	Moghadampour M, 2018
2015	Bangladesh	OXA-48	57.9%	Khatun R, 2015

1.13 Hypothesis

Null Hypothesis

Carbapenem resistance is not detected phenotypically by mCIM and Modified Hodge test. OXA-48 is not present in *Pseudomonas aeruginosa* isolates in population of Pakistan in a tertiary care hospital.

Alternate Hypothesis

Carbapenem resistance was detected phenotypically by mCIM and Modified Hodge test. OXA-48 gene is present in *Pseudomonas aeruginosa* strains in population of Pakistan in a tertiary care hospital.

1.14 Objectives of Research

The aim was to detect *Pseudomonas aeruginosa* isolate resistant to antibiotic carbapenem which is a drug of choice for these infections and to detect OXA-48 gene transcribing carbapenemase, previously not reported in Pakistan.

- To determine carbapenem resistant *Pseudomonas aeruginosa* phenotypically among in-door patients by Modified Hodge Test and mCIM.
- To study the presence of OXA-48, which exhibit carbapenem resistance in clinical isolates of *P aeruginosa.*

1.15 Problem Statement/ Problem of the study

OXA-48 gene is contributing to carbapenem resistance which is present in *Pseudomonas aeruginosa* isolates or strains cultured from different infection sites in Pakistan.

1.16 Significance of study

Multidrug resistance has come out as medical crises across the globe. In this regard, carbapenems are the last choice of defense against multi-drug resistance but regrettably resistance to carbapenem is on the rise in our country and other parts of the world. CR (Carbapenem resistant) *Pseudomonas aeruginosa* is identified more commonly in immune-compromised and hospitalized patients. It results in increased morbidity, mortality and prolonging the hospitalization. Phenotypic methods are valuable to detect

cabapenem resistance at an early step in the treatment plan. mCIM and Modified Hodge test are phenotypic methods which can guide the physicians to modify therapy in order to save patients lives. At molecular level, a multitude of genes transcribe carbapenem resistance (VIM, IMP, NDM, GES, KPC and OXA-48 gene which has not been reported in population of Pakistan). Bacteria have acquired noteworthy genetic diversity through acquisition of sequences from other related organisms. Horizontal gene exchanges create significant dynamic genomes in which significant amount of DNA are introduced into and detected from chromosomal matter. These lateral shifts have efficiently changed the pathogenic characters of bacterial species (Lozano C., 2018).

1.17 Operational Definitions

1) **Hospital acquired infection:** A hospital-acquired infection (HAI), also known as a nosocomial infection, is obtained in health care settings (Hospital or nursing home) and is not present at the time of admission. If the incubation period is unknown the signs and symptoms of infections that appear within 48 hours or more after admission are considered nosocomial. Infection is spread to the susceptible patient in the clinical settings by various means.

   Reference: Slimings, C., & Riley, T. V. (2013). Antibiotics and hospital-acquired Clostridium difficile infection: update of systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy*, *69*(4), 881-891.

2) ***Pseudomonas aeruginosa:*** *Pseudomonas aeruginosa* is the versatile "blue green pus forming bacteria" that has opportunity to infect people, especially those who are immune-compromised. It rarely causes infection in healthy persons but it is the principal cause of nosocomial infections.

   Reference: Finch, S., McDonnell, M. J., Abo-Leyah, H., Aliberti, S., & Chalmers, J. D. (2015). A comprehensive analysis of the impact of *Pseudomonas aeruginosa* colonization on prognosis in adult bronchiectasis. *Annals of the American Thoracic Society*, *12*(11), 1602-1611.

3) **Clinical and laboratory standards institute**: The clinical and laboratory standards institute (CLSI) is a volunteer-driven, membership supported, not for

earnings, standards development organization. CLSI supports the development and use of voluntary laboratory consensus standards and guidelines within the health care community.

Reference: Dudley, M. N., Ambrose, P. G., Bhavnani, S. M., Craig, W. A., Ferraro, M. J., Jones, R. N., & Antimicrobial Susceptibility Testing Subcommittee of the Clinical and Laboratory Standards Institute. (2013). Background and rationale for revised Clinical and Laboratory Standards Institute interpretive criteria (breakpoints) for Enterobacteriaceae and Pseudomonas aeruginosa: I. Cephalosporins and aztreonam. *Clinical infectious diseases*, *56*(9), 1301-1309.

4) **Carbapenem resistance:** Carbapenem resistance is a major and constant community health problem worldwide. It occurs mainly among Gram-negative microorganisms such as *Klebsiella pneumonae, Pseudomonas aeruginosa* and *Acinetobacter bauminnii* and may be intrinsic or mediated by transferable carbapenemase-encoding genes.

   Reference: Codjoe, F., & Donkor, E. (2018). Carbapenem resistance: a review. *Medical Sciences*, *6*(1),1.

5) **OXA-48:** OXA stands for oxacillinase and is a diverse group of β-lactamses classified to class D. Some of OXA β-lactamases additionally have the capability to hydrolyze carbapenems. Its production mediates resistance to pencillins and carbapenems.

   Reference: Glupczynski, Y., Evrard, S., Ote, I., Mertens, P., Huang, T. D., Leclipteux, T., & Bogaerts, P. (2016). Evaluation of two new commercial immunochromatographic assays for the rapid detection of OXA-48 and KPC carbapenemases from cultured bacteria. *Journal of Antimicrobial Chemotherapy*, *71*(5), 1217-1222.

CHAPTER 2

LITERATURE REVIEW

The significance of antibiotic use and the impact of antibiotic resistance have been discussed for last 70 years as per Table 5 on page 32. This resistance is not counteracted by manufacturing new antibiotics, so in these circumstances it is difficult to fight against infectious diseases (Van, Gouws & Hoffman, 2018). Antimicrobial resistance is matter of whole World because of its impacts on human-being health. Burden of infectious pathogen is threatening. Expenses of carbapenem resistant cases are 3,884 USD (Naylor et al., 2018).

Rizek et al., (2014) explained that carbapenemase genes are the major cause of carbapenem-resistant *Pseudomonas aeruginosa.* All cases were carbapenem resistant with MIC_{50} (64µg/ml), MIC_{90} (256µg/ml) to imipenem. Carbapenemase genes detected were blaSPM, bla_{KPC}, $bla_{VIM.}$ KPC gene exhibited carbapenem resistance in *Pseudomonas aeruginosa* can be determined by PCR. KPC gene can act as reservoir for transmission of resistance.

Castanheira, Deshpande & Costello (2014) explained the genetic relationship of carbapenem resistant *Pseudomonas aeruginosa,* this study showed metallo beta lactamase genes encoding VIM-2, VIM-4, VIM-1 and VIM-5, IMP-15, new Metallo-βLs (IMP-33, VIM-36 and VIM-37). Intrinsic resistance of carbapenem-non-susceptible *Pseudomonas aeruginosa* is troublesome in different European countries.

Caval Canti et al., (2015) explained multidrug resistance of *Pseudomonas aeruginosa* as a result of loss of OprD, over expression of efflux pumps and β-lactamase production. They determined the antimicrobial susceptibility by broth microdilution. Genes encoding β-lactamases, 16SrRNA methylase, aminoglycoside-modifying enzymes (AMEs), OprD and integron genes were detected by PCR (polymerase chain reactions). The blaSPM-1, blaKPC-2 and blaGES-1 genes were also identified in *Pseudomonas aeruginosa*. Frameshift mutations, premature stop codons and point mutations indicated the loss of

Table 5

Impact of antibiotic resistance

Development of antibiotics	**Development of antibiotic resistance**
1940---Sulfa drugs Penicillin Streotomycin	Penicillin-R-staphylococcus
1950-----Polymixins Tetracyclins Erythromycin	---------
1960----Vancomycin Methicillin Cephalosporin Nalidixic acid Gentamicin	Methicillin-R-staphylococcus aureus
1970-----Clindamycin	Penicillin-R-pneumonia Nalidixic acid-R-E-coli
1980-------Imipenem Ceftriaxone Ciprofloxacin	AmpC-cephalosporinases Extended spectrum β-lactamases
1990 ----------	Vancomycin-R-Enterococcus
2000------Linezolid Daptomycin Tigecyclin	Imipenem-R-Enterobacteriaceae Vancomycin-R-staphylococcus aureus
2010-------Ceftaroline	Pan-drug resistant Enterobacteriaceae

Reference Ventola C. L. (2015)

OprD. Loss of OprD was linked with over-expression of MexAB-opreM and MexXY-oprM. There were also cases of hyper production of AmpC. These relationships were determined by multilocus sequence typing. Ameen, Memon & Shaheen (2015) described

the imipenem resistance towards *P. aeruginosa* isolates. They explained MBL (metallo β-lactamase) production is the main culprit of IRPA (imipenem resistant *P. aeruginosa*). Imipenem cases were isolated by Kirby Bauer Diffusion technique. MBL (metallo β-lactamase) production was detected by EDTA (Ethylenediamine tetraacetic acid) combined disk test. Alongwith it polymyxin B resistance is alarming for health care set-ups. There is trepidation of pan-drug resistant *P.aeruginosa* followed by multi-drug resistance. We are waiting for bacteriophage therapy and molecule like MBL inhibitors therapy. Yayan, Ghebremedhin and Rasche (2015) highlighted the multi-drug resistance of *Pseudomonas aeruginosa*. There are two types of pneumonia, community acquired and hospital acquired pneumonia. Major cause of both pneumonias is *P. aeruginosa*. They presented 54.2% pneumonia was community acquired and 45.8% by hospital acquired. *P. aeruginosa* exhibited resistance towards fosfomycin, ciprofloxacin, levofloxacin, ceftazibime, piperacillin, tazobactam, tobramycin, gentamicin, meropenem, cefepime and amikacin. But nothing was resistant to colistin. Terzi, Kulah, Atasoy and Ciftci (2015) discussed the mechanism of resistance of imipenem through oprD. Basically low level of OprD is responsible for both carbapenem and non-carbapenem resistance of *P. aeruginosa*. Molecular level of resistance was determined by real-time polymerase chain reaction (qPCR).

Jilu, Yaping, and Fang (2015) explored the association of outer membrane protein OprD2 and carbapenem resistance in isolates of *P. aeruginosa*. Minimum inhibitory concentration of meropenem and imipenem was determined by Agar dilution methods in isolates of *P. aeruginosa.* OprD2 gene was tested by PCR and DNA sequencing. Resistance towards carbapenem like imipenem and meropenem was caused by fragment deletion and fragment insertion. They used SDS-PAGE to highlight the outer-membrane protein in isolates of CRPA (carbapenem resistant *Pseudomonas aeruginosa*). They determined mutation or loss of the OprD2 was responsible for resistance. Mona Shaaban et al., (2015) demonstrated the different mechanism of carbapenem resistance in isolates of *P. aeruginosa*. They explained the role of MexB and OprD in resistance. They also detected VIM-1 and VIM-2 (Verona integron-encoded-metallo-β-lactamase) and NDM enzymes and relatedness with resistance of carbapenem in isolates of *P. aeruginosa.* Dortet et al., (2015) described the carbapenemase-producing *P. aeruginosa* and

Enterobacteriaceae. All carbapenemase genes like NDM-1, KPC, OXA-48, VIM-4, and VIM-2 were situated on self-conjugative plasmids.

Shaikh et al., (2015) illustrated ESBLs (extended spectrum β-lactamase) resistance in isolates of *P. aeruginosa* and *Enterbacteriaceae*. They explained different mechanisms and genes which were responsible for resistance.

Kariem et al., (2015) stated ESBLs (extended spectrum β-lactamaces) which exhibited resistance in Gram negative bacteria towards third generation cephalosporins. *Enterobacteriaceae* and *Pseudomonas aeruginosa* were serious threats to critical patients in the last century. New Delhi MBL (NDM) was first time discovered in Tunisia in female patient. This gene was detected in *Klebsiella pneumonae* microorganism.

Mathlouthi et al., (2015) showed the molecular mechanism of carbapenem resistance in *P. aeruginosa* and *Acinetobacter baumannii.* All imipenem resistant *P. aeruginosa* presented OprD mutation. Gene involved in resistance was blaVIM-2. The bla_{OXA-23} and the bla_{OXA-24} genes were responsible for carbapenem resistance in *Acinetobacter baumannii*. Bla_{OXA-48} gene also exhibited resistance towards carbapenem.

Sahuquillo et al., (2015) told extended spectrum cephalosporinases and β-lactamases. These carbapenem antibiotics were produced from natural bacterium Streptomyces cattleya. Imipenem was derived from thienamycn in 1985. After that carbapenem-resistant organisms like *Ecoli, Klebsiella pneumoniae, P. aeruginosa* and *Acinetobacter baumannii* were introduced. Different mechanisms were involved in resistance, the most common was carbapenemase. These enzymes blocked β-lactam ring of carbapenem antibiotics and attained by horizontal-gene-transfer. Plasmids, integron and transposons were indulged in antibiotic resistance. This resistance leads to high mortality rate in critical patients.

Khataminejad et al., (2015) explained Fe_2O_3 nanoparticles can enhance the susceptibility of imipenem. This characteristic was tested on MBL *Pseudomonas aeruginosa*. Farzana & Shamsuzzaman (2016) explained the synergistic effects in imipenem resistant *Pseudomonas aeruginosa* isolates. Carbapenem (imipenem and meropenem) resistance

was also found out by disc diffusion method. This study indicated imipenem-colistin with 50% synergism, imipenem-ceftriaxone with 30% synergism and piperacillin/tazobactum-imipenem with 20% synergism. Kateete et al., (2016) described the multidrug resistance *P. aeruginosa* and *Acinetobacter baumannii.* In this study they determined the prevalence of carbapenem-resistant *Pseudomonas aeruginosa, Acinetbacter baumannii* in Uganda. In Uganda, prevalence rate of carbapenem-resistant *P. aeruginosa* and *Acinetobacter baumannii is* stumpy as compare to South-East- Asia. Al-Agamy et al., (2016) discussed molecular and prevalence rate of antibiotic resistance in *P. aeruginosa.* 34 cases of carbapenem were detected from samples of tertiary hospital in Riyadh, Saudi Arabia. All cases exhibited resistance towards ceftazidime. 15 cases showed carbapenem resistance. Genes were detected for carbapenem, VIM-1, VIM-2, VIM-4, VIM-11, VIM-28, and IMP-7. 5 isolates presented with MexAB gene. 34 carbapenem-resistant *P. aeruginosa* were analyzed for PFGE that divulged 14 different pulsotypes. So carbapenem-resistance showed diversity of mechanisms.

Tada et al., (2016) described the IMP-type metallo-β-lactamase. Complete genome sequencing was done that presented two copies of $bla_{IMP-34.}$ It was situated on class -1 integron, tnp A (Spa7)-intl 1-qacG-bla_{IMP-34}-acc (6')-Ib-qacEdelta-1-sul-1_orf5-tni-βdeltatniA.

Pragasam et al., (2016) described the surveillance of AMR (antimicrobial resistance) of *P. aeruginosa* and *Acinetobacter baumannii.* Basically the Government of India and Indian Council of Medical Research was involved on back of this study. They found out the antibiotic sensitivity, ESBL (extended spectrum β-lactamases) and carbapenemases of *Pseudomonas aeruginosa* and *Acinetobacter bauminnii.* They detected ESBLs, $bla_{VEB,}$ $bla_{TEM,}$ and bla_{shv} in *P. aeruginosa* and $bla_{PER,}$ bla_{TEM} and bla_{SHV} in *Acinetobacter bauminnii*. The most prevalent carbapenemases $bla_{VIM,}$ $bla_{NDM,}$ bla_{GES} and bla_{IMP} in *Pseudomonas aeruginosa* and $bla_{OXA-23,}$ $bla_{OXA58,}$ bla_{NDM} and bla_{VIM} in *Acinetobacter bauminnii* were the most prevalent carbapenemases. Bla_{OXA-51} gene was intrinsic to *Acinetobacter bauminnii*. They proposed that plasmid–mediated mechanisms of resistance are low than chromosomal-mediated resistance. Especially class D oxacillinases are more popular in *Acinetobacter bauminnii.*

Judd et al., (2016) described the financial and clinical ending of emergence of carbapenem resistance. Meropenem resistance was the major analyst of mortality.

Pragasam et al., (2016) described the multi-drug resistance of *Pseudomonas aeruginosa.* Carbapenems were the last choice of multi-drug resistant *Pseudomonas aeruginosa.* Intrinsic/chromosomal resistance towards carbapenems participates in emergence of resistance to carbapenem. They observed imipenem was resistant but meropenem was susceptible, on other hand meropenem was resistant and imipenem was susceptible. At molecular level they detected imipenem resistant meropenem susceptible as a result of mutation of OprD gene and over-expression of MexAB efflux pumps.

Meletis (2016) illustrated the carbapenem resistance in Gram negative bacteria. In this study, they explained the negative impact of this resistance in future, because multidrug resistance can lead to pan-drug resistance. As a result, mortality rate will be increased followed by poor infection control.

Fan et al., (2016) explained the epidemiology of multidrug resistant *P. aeruginosa* in mainland China. 233 carbapenem-resistant isolates were detected at molecular level. IMP-type enzymes, VIM-2, KPC-2 genes were detected from isolates. Genetic diversity can be determined by MLST. ST292/PFGE genotypic detected XDR (extensively drug resistance). They explained MDRPA (multidrug resistant *Pseudomonas aeruginosa*) progressed as a result of diversity change.

Tada et al., (2016) explained multidrug drug resistance of *P. aeruginosa* Vietnam. Bla_{IMP-51}, blaIMP-26, blaIMP-15, and blaNDM-1 were detected. BlaIMP-26 was related with ST235 and blaIMP-26 was resistant to doripenem and meropenem as a result of increased hydrolytic activities. Li et al. (2016) described the XDR-PA (extensively drug resistance *P. aeruginosa*) during 2011-2012. Hashem et al., (2016) explained that β-lactamases (BML) was responsible for resistance of *P. aeruginosa*. This study observed multidrug resistance of *P. aeruginosa* towards cefotaxime, ceftazidime, cefepime, aztreonam and meropenem.

Swamin athan et al., (2016) narrated the carbapenem resistance of Gram-negative bacteria especially *P. aeruginosa.* Carbapenemase production was checked by CDDT,

MHT and Etest. Uniplex PCR was used for New Delhi metallo-β-lactamase-1 for *Ecoli* and *Klebsiella pneumonae*. Multiplex PCR was used for detection of imipenemases and Verona imipenemase in *P. aeruginosa* and *Acinetobacter baumannii*. Gram-negative bacilli exhibit resistance towards carbapenem.

Tarashi et al., (2016) explained that *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were major risk factors for hospital acquired infections. They established the frequency of blaIMP, blaVIM, blaDIM, blaAIM, blaGIM and blaNDM genes. Different genes of resistance were detected by PCR and sequencing techniques. Metallo-β-lactamase (MBL) was produced by CDDT (combined disc diffusion test).

Baran and Aksu (2016) explained the carbapenem resistance of Enterobacteriaceae. OXA-48 was responsible for resistance of *Enterobacteriaceae*.

Simsek et al., (2016) correlated the mortality rate with multi-drug resistance of *P. aeruginosa.* Imipenem, amikacin, tobramycin, ciprofloxacin, piperacillin, piperacillin/tazobactam, ceftazidime and cefepime resistance rate of *P. aeruginosa* was detected according to CLSI (clinical laboratory standard institute). They noticed multi-drug resistance in *P. aeruginosal i*nfections.

Sonmezer et al., (2016) explained resistance of antibiotics towards *P. aeruginosa* that was responsible for high morbidity and mortality rate. In this study six antibiotics like (imipenem, meropenem, piperacillin-tazobactam, ciprofloxacin, amikacin and ceftazidime) were taken as univerate.

Jabalameli et al., (2016) explained the mortality rate among burn patients as a result of carbapenem resistant *P. aeruginosa*. Different genes VIM (Verona integron-encoded Metallo-beta lactamase, imipenemase (IMP), Sao Paulo metallo-beta lactamase (SPM), German imipenemase (GIM), New Delhi metallo-beta lactamase (NDM), Dutch imipenemase (DIM), Adelaide imipenemase (AIM), Seoul imipenemase (SIM), Serratia metallo-beta lactamase (TMB) and Florence Imipenemase (FIM) were detected. CRPA (Carbapenem resistant *Pseudomonas aeruginosa)* had less information related with frequency and prevalence.

Ansari et al., (2016) explained that *P. aeruginosa* is a normal flora of 10% of colon, but whenever it has chance, it can spread infections. It can spread infection in those people whose immunity is compromised. It exhibits resistance towards antibiotics as a result of enzyme beta-lactamases. In laboratory, isolates of *P. aeruginosa* were checked for antibiotic susceptibility by Kirby-Bauer-Disc diffusion method according to CLSI (clinical and laboratory standard institute). Resistance towards antibiotics was high as a result of beta lactamase enzyme.

Olga et al., (2016) described the antibiotic resistance of *P. aeruginosa* in natural environment. They collected 245 isolates from several aquatic sites, in Greece. Antibiotic resistance patterns of cefotaxime-aztreonam-ceftazidime, cefotaxime-aztreonam-meropenem, cefotaxime-ceftazidime-meropenem, cefotaxime-ceftazidime-aztreonam-meropenem, cefotaxime cefotazidimeaztreonam-meropenem, cefotaxime-ceftazidime-cefepime-aztreonam meropenem were checked for ESBLs (extended spectrum beta-lactamases). In 77 isolates out of 245 possessed class I and class II integrase genes. They concluded *P. aeruginosa* in Greek water could act as source of antimicrobial resistance and harmful for humans and animals.

Baran and Aksu (2016) discussed that *Enterobacteriaceae* are causative factors of serious community –acquired and hospital acquired infections. The whole world has to face CRE (carbapenem resistant *Enterobacteriaceae).* They are going to increase morbidity and mortality rate. They determine carbapenem resistance both phenotypically and genotypically. Antimicrobial susceptibility rate was determined by vitek-2 system. Resistance towards meropenem and ertapenem was checked by MHT (modified Hodge test). Metallo-beta lactamase production was determined by E-test MBL strips. Carbapenem resistance genes (IMP, VIM, KPC, NDM-1 and OXA-48) were detected by multiplex PCR. They took 181 CRE isolates. 86 isolates out of 181 exhibited OXA-48 genes for carbapenem resistance. Only one VIM gene was detected. Three isolates showed both OXA-48 and NDM-1 and only one isolate showed both OXA-48 and VIM. 92 strains exhibited multidrug resistance. They brought to close with OXA-48 is majorly responsible for carbapenem resistance in *Enterobacteriaceae* family in hospitalized patients.

Duin and Doi (2017) explained antimicrobial resistance as a result of plasmid-mediated genes. They mentioned Ambler classification of beta lactamases. Class A exhibits gene (KPC Klebsiella pneumonia carbapenemases), class B exhibits genes (metallo beta lactamases including NDM new Delhi metallo beta lactamases), and class D exhibits gene (oxa-48 like carbapenemases). OXA-48 gene exhibits antimicrobial resistance commonly in Turkey and surrounding countries.

Yucel et al., (2016) described the carbapenem resistant *P. aeruginosa*. Carbapenemase genes like (IMP, VIM, and NDM) are responsible for carbapenem resistance. They took 48 isolates of *P. aeruginosa* from blood samples of hospitalized patients. MBL production was checked with E-test. Genotypically these genes were detected by PCR. *P. aeruginosa* exhibited 87.5% resistance towards carbapenem. They detected VIM firstly as carbapenem resistant gene in *P. aeruginosa* in Turkey.

Buehrle et al., (2017) explained the multidrug resistance of *P. aeruginosa*. 37 patients showed carbapenem resistance towards *Pseudomonas aeruginosa.* Mortality rate was 19%. Goncalves et al., (2017) described the characteristic and resistance towards antibiotic therapy of *P. aeruginosa*. *P. aeruginosa* is responsible for hospital acquired infections. It gave the challenges of resistance towards therapy and virulence factors. Two main factors like enzyme metallo beta lactamase and production of biofilm were responsible for bacteremia by carbapenem resistant *P. aeruginosa*. This was case control study, done at Uberlandia Federal University Brazil. Genes were detected by PCR (Polymerase chain reaction). Biofilm production was checked by quantitative tests. This study concluded that 73.9 % were multidrug resistant, 43.9 % were resistant to carbapenem. Bla_{SPM} gene and bla_{VIM} gene were detected. Mortality rate was high with bacteremia by carbapenem resistant *P. aeruginosa.* These were commonly present in those patients who were on ventilators. Mirsalehian et al., (2017) explained the carbapenem resistant *P. aeruginosa*. Carbapenem was last resort in multidrug resistant cases of *P. aeruginosa*. They explained the mechanisms of carbapenem resistance in burn patients. Over production of AmpC was detected phenotypically and carbapenemase gene was detected by PCR. PCR sequencing was used to find out the mutation transcription level of OprD. Two genes like bla_{VIM} bla_{IMP} showed resistance toward imipenem and

meropenem. Rostami et al., (2018) described carbapenem resistance of *P.aeruginosa* is opportunistic organism and responsible for infections in hospitalized patients. They found out frequency by four main mechanisms of resistance like metallo beta lactamases productions (blaIMP, blaVIM, blaSPM and blaNDM). Overproduction of Mex AB-OprM and MexXY efflux pumps, overproduction of AmpC beta lactamase, reduced OprD expressions). They took 107 isolates of *Pseudomonas aeruginosa* from burn patients. Carbapenemase was checked by E test. Genes were detected by PCR. Carbapenem resistant cases were determined by real time PCR. 78.5% isolates were resistant to imipenem, 46.7% were resistant to meropenem and 15% to doripenem. Carbapenem resistant *P. aeruginosa* was serious matter in burn patients. Dantas et al., (2017) explained multidrug resistance and extensively drug resistance of *P. aeruginosa*. This resistance was related with over-expression of efflux pumps, AmpC over-production and loss of OprD. This was retrospective study. Genes were detected by quantitative and qualitative PCR and isolates typing was done by pulse field gel electrophoresis. *P. aeruginosa* was related with preceding use of carbapenem and trachiostomy. BlaSPM-1 and bla_{VIM} genes were detected. 71.4% were resistance and presented by loss of OprD porin, 57.1% by MexABOprM, 64.3% MexXY. They concluded intrinsic resistance was culprit for these. Campana et al., (2017) explained carbapenem resistance and cephalosporins susceptibility. This study was conducted in Sao Paulo Brazil. Genes were detected by PCR and then DNA sequencing was done. Carbapenem hydrolysis activity was determined by spectrophotometers and MALDI-TOF assays. Transcription level of OprD was checked by Qrt-PCR. In this study they explained several chromosomal mechanisms of carbapenem resistance. Shaaban et al., (2017) discussed the carbapenem resistance of *P. aeruginosa*. They explained different mechanisms which are involved in carbapenem resistance. Antimicrobial susceptibility was determined by disc diffusion methods. MexB and OprD were detected by real time PCR. 16 isolate showed carbapenem like imipenem and meropenem resistance. VIM-1, VIM-2 and NDM genes which exhibited antibiotic resistance were detected by PCR (polymerase chain reaction). All these mechanisms of resistance will reduce the treatment options.

2.1 Carbapenem resistance of *Pseudomonas aeruginosa* in China

Zeng et al. (2016) explained carbapenem resistance of *Pseudomonas aeruginosa.* Ceftazidime and cefipime were susceptible to *P. aeruginosa*. Carbapenem resistance was associated with OprD. The sequencing of OprD gene was done that was related with multiple point mutations, large fragments, substitutions, deletions and insertions. Expression of genes was determined by real-time PCR. Loss of OprD and overexpression of MexXY-OprM and MexAB-OprM was susceptible for carbapenem resistance in cephalosporin-sensitive *P.aeruginosa.*

2.3 Antibiotic resistance of *Pseudomonas aeruginosa* in Pakistan

Samad et al., (2017) found out the prevalence and susceptibility pattern of *Pseudomonas aeruginosa. Pseudomonas aeruginosa* exhibits multidrug resistance in patients of respiratory tract infections. They collected 615 samples of sputum and grew them on blood agar, MacConkey agar and chocholate agar. Antibiotic sensitivity was determined by Kirby Bauer's disc diffusion according to CLSI. Extensive drug resistance was 25% and pan drug resistance was 10.71%. Cefoperazone+salbactum, piperacillin+tazobactum were resistant antibiotics. Ali et al., (2015) found out antimicrobial resistance of *P. aeruginosa* in hospitalized patients. These isolates were analyzed by 20NE and antibiotic sensitivity was determined by Kirby Bauer disc diffusion method. They exhibited resistance as Ofloxacin 61.3%, cefepime 57.3%, ceftazidime 53.9% and Amikacin 53%.

2.4 In other countries

Sumoto et al., (2017) described the role of cefederol in cases of carbapenem resistant Gram negative rods like *P. aeruginosa*, *Klebsiella pneumonae* and *Acinetobacter baumannii.* They performed experiments on immunocompetent rat models with respiratory tract infections, then recreating plasma pharmacokinetics profile in human beings. They took six clinical isolates one cephalosporin susceptible *P. aeruginosa*, one multidrug resistant *Pseudomonas aeruginosa*, two multidrug resistant *Acinetobacter Bauminnii*, two carbapenem resistant *klebsiella pneumonae.* They concluded cefederol killed the carbapenem resistant isolates in lungs. Cefederol was effective in carbapenem resistant *P. aeruginosa*, Acinetobacter bauminnii and Klebsiella pneumonae isolates.

Yadar et al., (2017) determined the multidrug resistance of *P. aeruginosa*. This problem could be overcome by combination of antibiotics. They used single drug therapy and combination of imipenem with tobramycin or amikacin. Imipenem with combination of tobramycin or amikacin was effective with resistant cases. Aminoglycoside was used to increase the efficacy of imipenem.

2.5 Carbapenem resistant *Pseudomonas aeruginosa* in India in 2017

Rehman et al., (2018) explained NDM (New Delhi metallo beta-lactamase) related carbapenem resistance of *P. aeruginosa* and *Acinetobacter bauminnii*. They concluded 29.23% resistant isolate of *P. aeruginosa* and 18.8% of *Acinetobacter bauminnii* toward carbapenem. Genes blaNDM-1 and blaNDM-2 were detected. Mishra et al., (2017) described the characteristics of *P. aeruginosa*. Carbapenem is the last resort in penicillin and cephalosporin resistant cases. They determined imipenem resistance towards carbapenem in septicemic patients especially neonates. They used vitek-2, CDST (Combined disc synergy test), EDTA (CDST-IPM) and DDST (double disc synergy test). *P. aeruginosa* were collected from endotracheal tube and pus. Imipenem-resistant *P. aeruginosa* were susceptible to piperacillin-tazobactum. Choudhury et al., (2017) explained OprD-porin associated carbapenem resistance of *P. aeruginosa* from tertiary care hospital of Northeast India. Sequencing determined mutation patterns. Along with mutation, decreased expression of Opr D gene was responsible for resistance of imipenem and meropenem.

2.6 Carbapenem resistant *Pseudomonas aeruginosa* in turkey in 2017

Malkocoglu et al., (2017) determined carbapenemase production in carbapenem resistant *P. aeruginosa*. Genes like bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{OXA-48}, and bla_{GES} were determined by PCR.

CHAPTER 3

METHODOLOGY

3.1 Research Design

Observational cross-sectional-study

3.2 Ethical approval

The proposed research had been reviewed and approved by Ethical Committee of FRC (Faculty Research Committee), ERC (Ethical Review Committee) (Bahria University Medical and Dental College) and Ethical Review Committee of PNS Shifa Hospital (as given in Appendices Aand B).

3.3 Setting

Pus Samples were received from various wards (Medicine, Surgery, ENT, Burn Unit, Plastic Surgical Ward, Family Officer Ward, and ICU (Intensive Care Unit) at PNS Shifa Hospital.

3.4 Inclusion criteria

All *P. aeruginosa* were isolated from pus samples of hospitalized patients. These specimens were collected from infected ear, infected burn wounds, diabetic foot ulcers, infected plastic surgery flaps and so on. Specimens of all age group and both gender were received from various wards at PNS Shifa Hospital. Phenotypically all specimens screened for carbapenem resistance and only carbapanem resistant cases were used for detection of OXA-48 genes by real time PCR.

3.5 Exclusion criteria

Repeat samples of *P.aeruginosa* and outdoor patients were excluded. OXA genes other than OXA-48 were excluded.

3.6 Duration of study

September 2018-May 2019

3.7 Sample size estimation

The sample size was calculated by using the method of sample size with 95% confidence interval, 5% margin of error and prevalence of 10.2% according to WHO calculator.

Level of confidence measure is 1.96, margin of error 0.05, baseline levels of indicators 10.2% (Lin et al., 2016).

$$n = \frac{3.8416*(0.1*(1-0.1)}{0.5*0.5} = 140$$

Reference: Lin, K. Y., Lauderdale, T. L., Wang, J. T., & Chang, S. C. (2016). Carbapenem-resistant Pseudomonas aeruginosa in Taiwan: Prevalence, risk factors, and impact on outcome of infections. *Journal of Microbiology, Immunology and Infection*, *49*(1), 52-59.

3.8 Sampling technique

Non probability convenient sampling

3.9 Human subjects and consent

Pus samples of hospitalized patients in PNS Shifa Hospital Karachi were included. Information was obtained about demographic characteristics like all age groups and both genders.

Consent was taken in form of thumb impression and signature after verbally explaining about project. In case of children consent was taken from their parents (as given in Appendices C).

3.10 Materials

a. Clinical samples

Clinical samples included as pus from ear infections like otitis media, infected burn wounds, diabetic foot ulcers and infected surgical wounds of admitted patients at PNS Shifa.

b. Culture Media

Blood agars, MacConkey Agar, Muller Hinton agar were used.

c. Disc

Meronem (10µg) (oxoid), Imipenem (10µg) (oxoid) were used.

d. Equipment

Real time PCR (Polymerase chain reaction)

Qiamp pathogen mini Kit Performa

Briogene Pvt Ltd Company

e. Performa

As mentioned in Apendices D

3.11 Lab. Parameters

Parameters of study included identity number of hospital, age, gender, samples of patients.

3.12 Methodology/ Protocol

Isolation and preservation of *Pseudomonas aeruginosa* from clinical samples

After the isolation of *Pseudomonas aeruginosa* colonies, these organisms were preserved in nutrient broth with glycerol in a volume of 2ml in screw capped bottles.

Identification of isolates

We used three methods for identification of isolates of *Pseudomonas aeruginosa*.

- Conventional methods
- Phenotypic methods
- Genotypic methods

- **Conventional identification**

These included the following parameters.

1. Colonial characterization

Pseudomonas aeruginosa was grown on blood and MacConkeys agar at 35±2^{o}C for 24 hours under aerobic conditions. It produced colonies with smooth, mucoid and irregular borders.

PIGMENTATION

This organism produced mostly green and black pigments.

Gram staining

Procedure

Primary stain (crystal violet) was added to fixed smear of bacterium as per Figure 9 on page 47. Iodine was added; made a crystal violet-iodine complex. Decolorizer was added like ethanol, followed by safranin (counterstain).

Blood agar

Nutritional requirement of *Pseudomonas aeruginosa* is very simple. In the laboratory, it required acetate as a source of carbon and ammonium sulphate as a resource of nitrogen. *Pseudomonas aeruginosa* growth was appeard as typical metallic sheen on blood agar plate as per Figure I0 & 1I on page 48. It produced large, smooth with flat borders, mucoid and raised colonies.

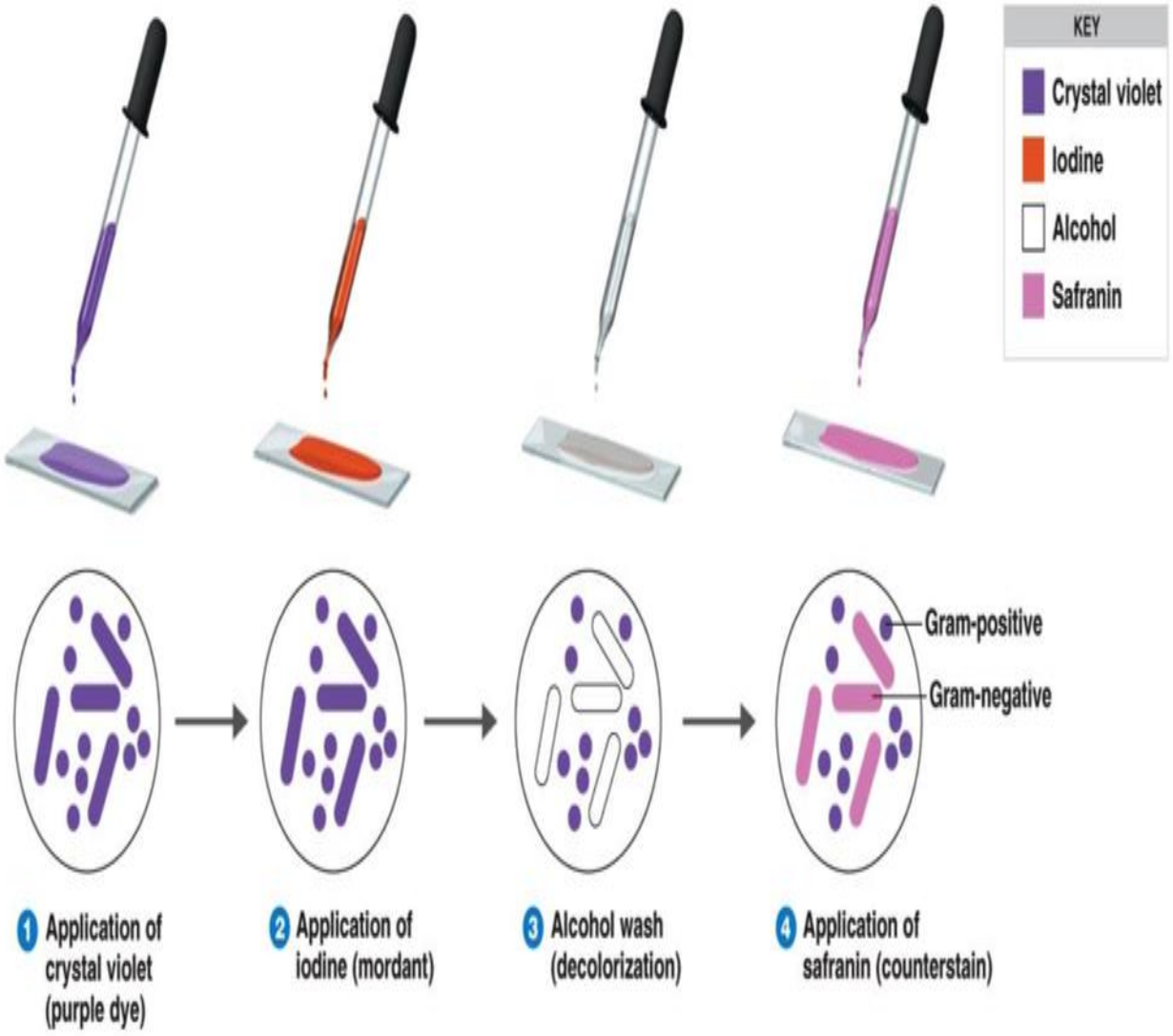


Figure 9 Procedure of Gram Staining (Becerra, S. C et al., 2016)

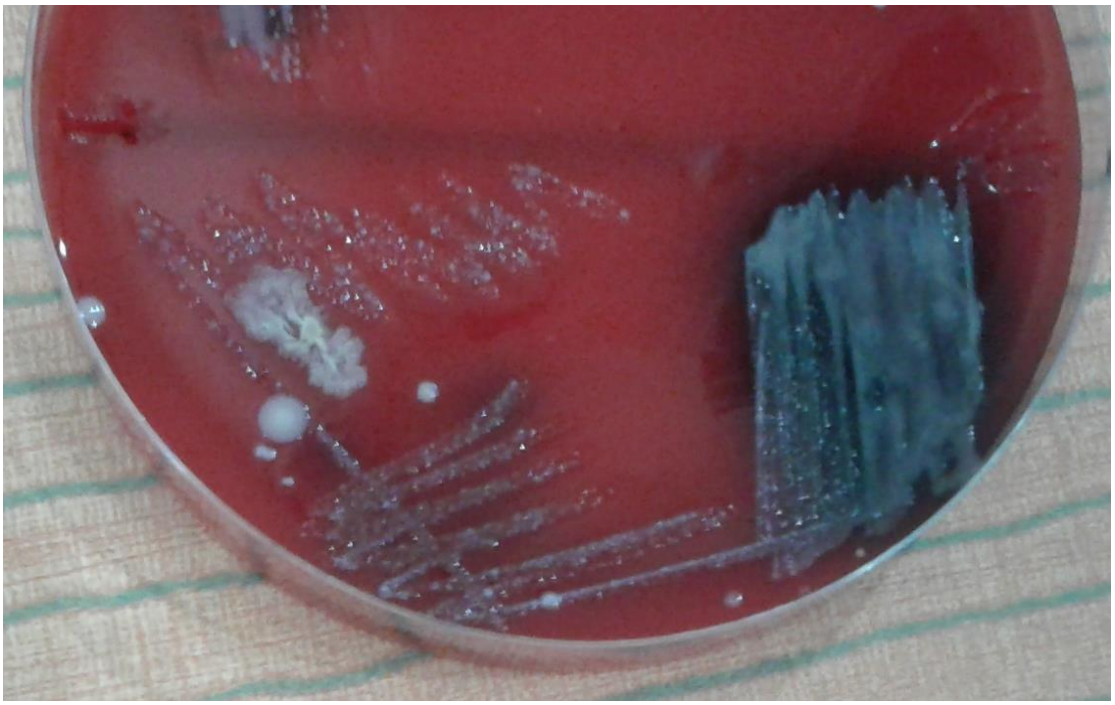


Figure I0 Growth of *Pseudomonas aeruginosa* on Blood Agar (inside plate)

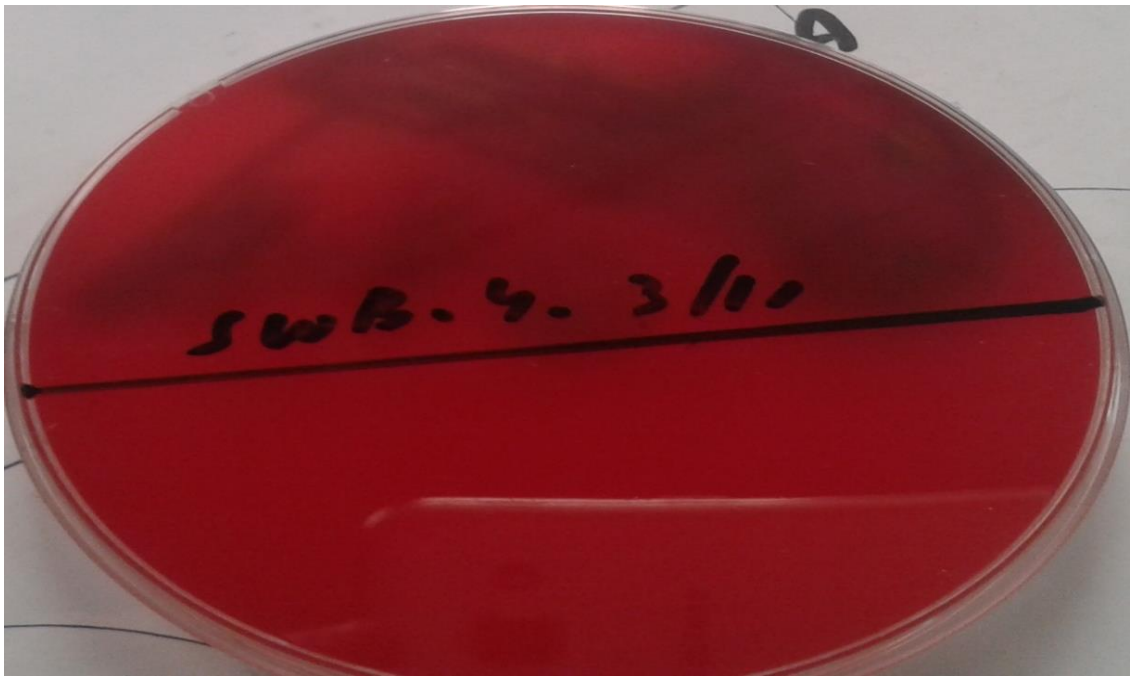


Figure 1I Growth of *Pseudomonas aeruginosa* on blood agar (outside plate)

MacConkey Agar

Pseudomonas aeruginosa gave colourless colonies on MacConkey agar at 35±2°C for 24 hours as it is non lactose fermenter as per Figure 12 & 13 on page 50.

Oxidase test

It is a biochemical test; it indicated the presence of cytochrome oxidase. Basically *Pseudomonas* aeruginosa possessed this enzyme; it reduced colorless reagent by using tetra-methyle-P-phenylenediamine dihydrochloride (or KOVACS reagent). This reagent was oxidized by cytochrome c; it turned to dark purple color as per Figure 14 on page 51.

Filter Paper Test

We used filter paper test. A small piece of filter paper was soaked in 1% Kovács oxidase reagent and let it dry. A well-isolated colony was picked with help of a loop from a fresh (18- to 24-hour culture) bacterial plate and rubbed onto treated filter paper. We noticed Color changes within few seconds. It indicated that this test was positive for *Pseudomonas aeruginosa* as per Figure 15 on page 51.

Antibiotic susceptibility disc diffusion method

In case oxidase test was positive, antimicrobial susceptibility testing was checked by disc diffusion method on Mueller–Hinton (MH) agar plate as per CLSI 2019(Clinical laboratory standard international) guidelines. We selected a pure culture plate of isolated organism, and then colony of isolated organism was aseptically emulsified in the sterile saline solution. We took a sterile swab and dip it into broth culture of organism. We gently squeezed the swab and streak it on Muller Hinton Agar. Then we applied two antibiotics imipenem (oxoid) (10µg) and meropenum (oxoid) (10µg) with help of forceps (as per Figure 16 on page 52, Figures 17 and 18 on page 53). We incubated it at 35±2°C for 24 hours and checked its zone according to CLSI. When zone was equal or less than 15mm in diameter, it showed resistance both for imipenem and meropenem. Diameter of zone 16-18mm was considered as intermediate. Diameter, equal or more than 19mm, indicated sensitivity for imipenem and meropenem.

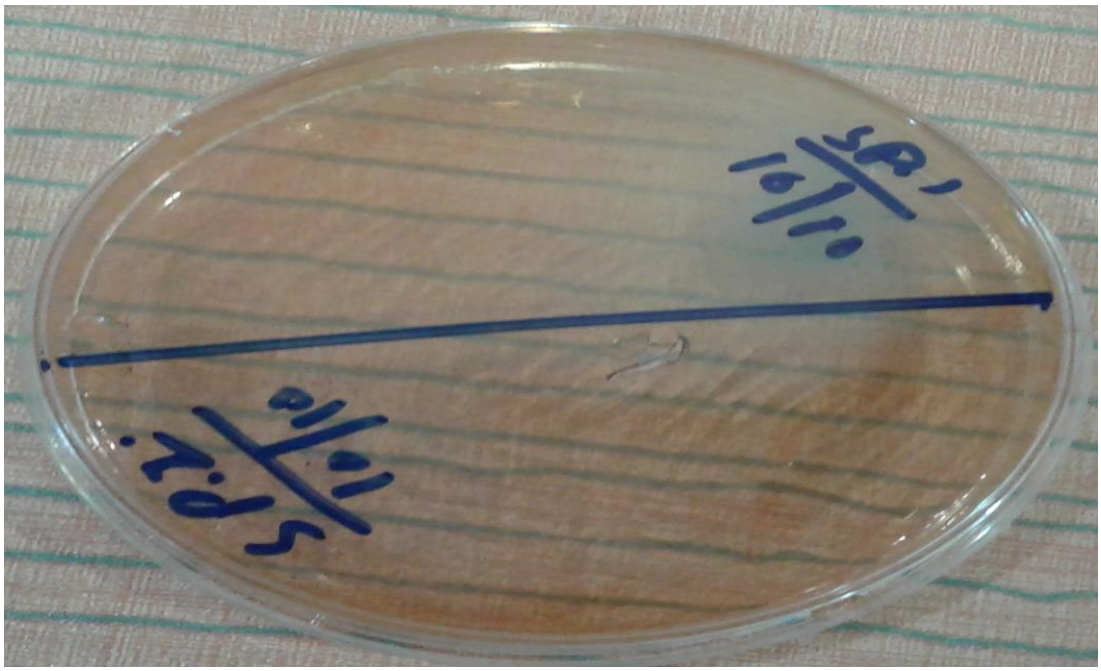


Figure 12 Growth of *Pseudomonas aeruginosa on* MacConkey (out side plate)

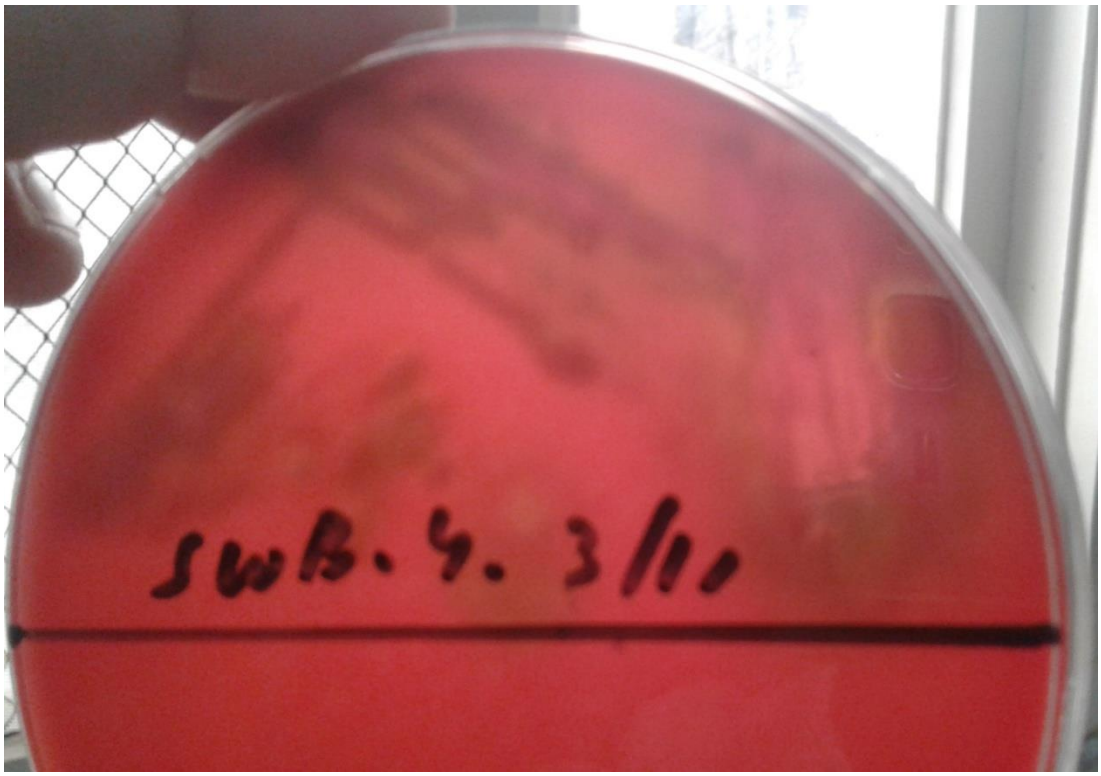


Figure 13 Growth of *Pseudomonas aeruginosa* on Mac Conkey agar

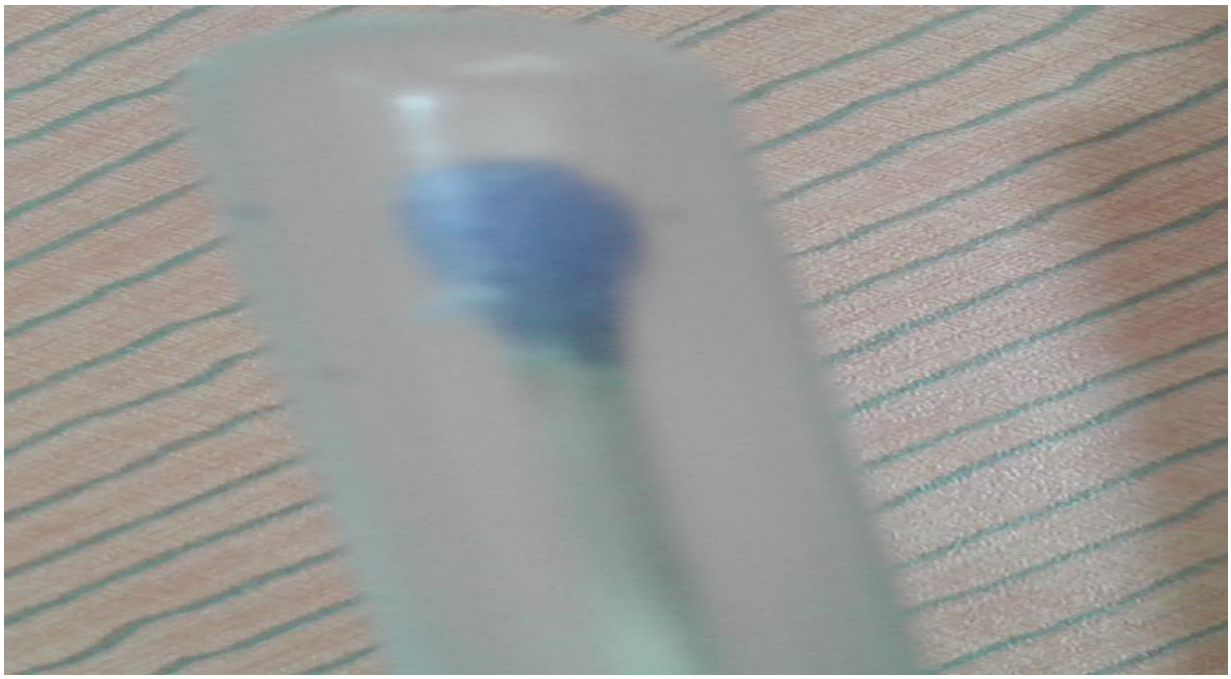


Figure 14 Positive oxidase test (swab stick method)

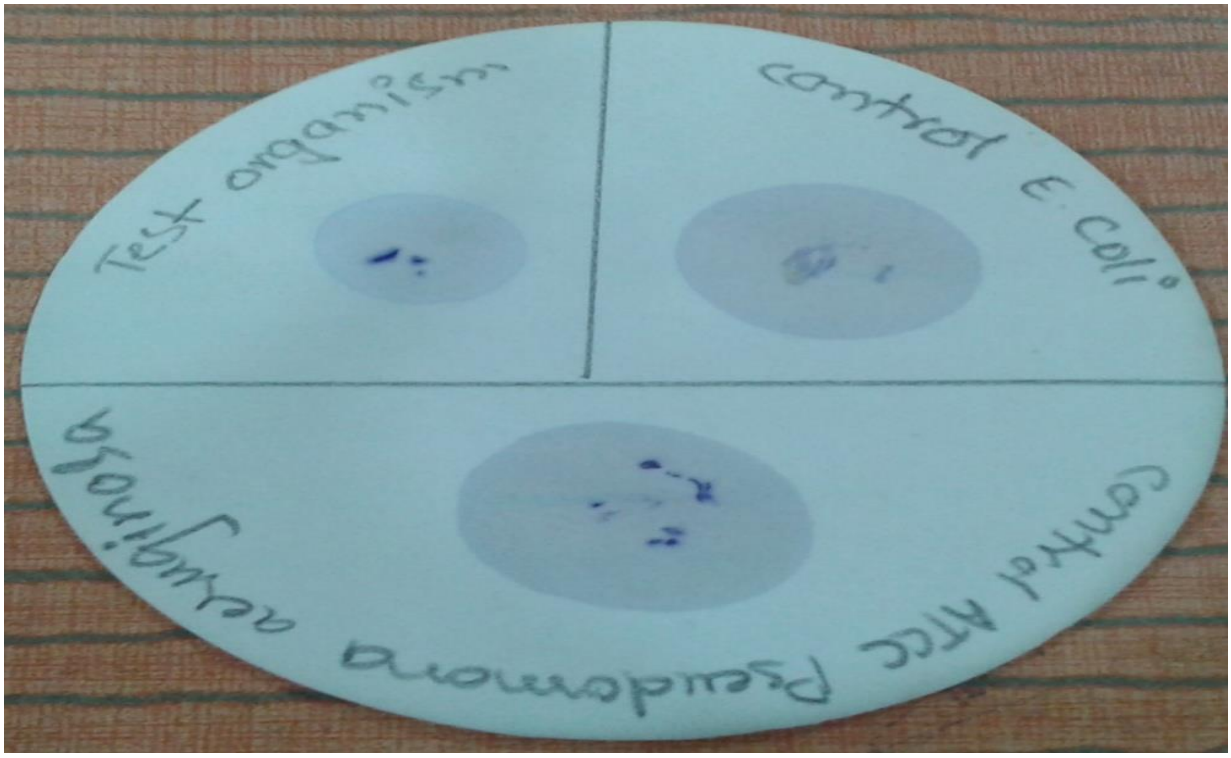


Figure 15 positive and negative oxidase test (filter paper method)

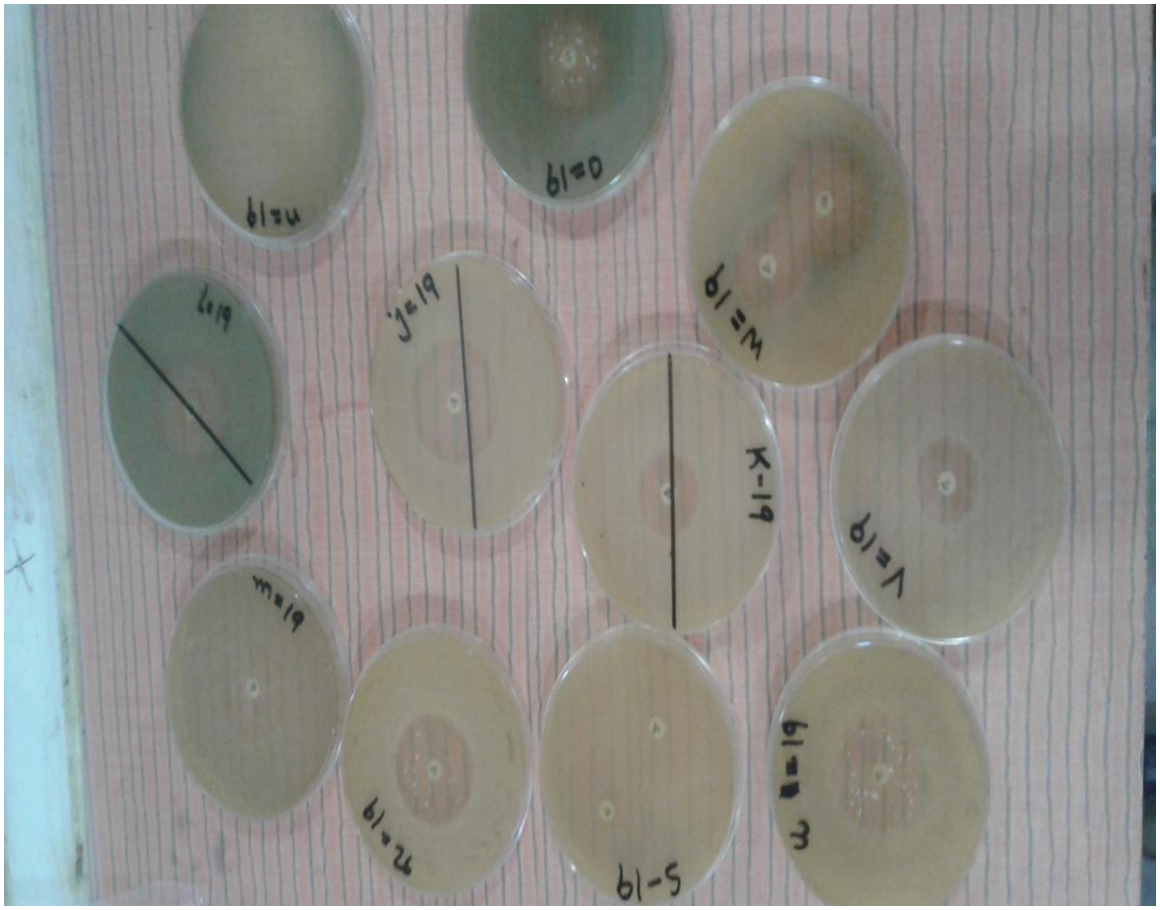


Figure 16 Antibiotic sensitivity disc methods

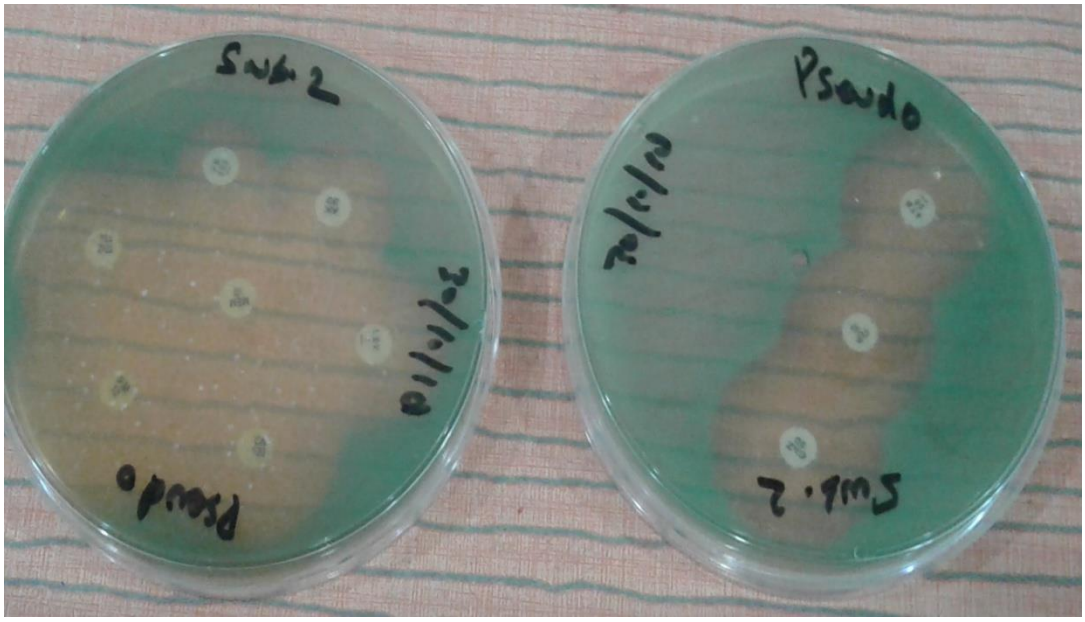


Figure 17 Antibiotic sensitivity disc methods (sensitive case)

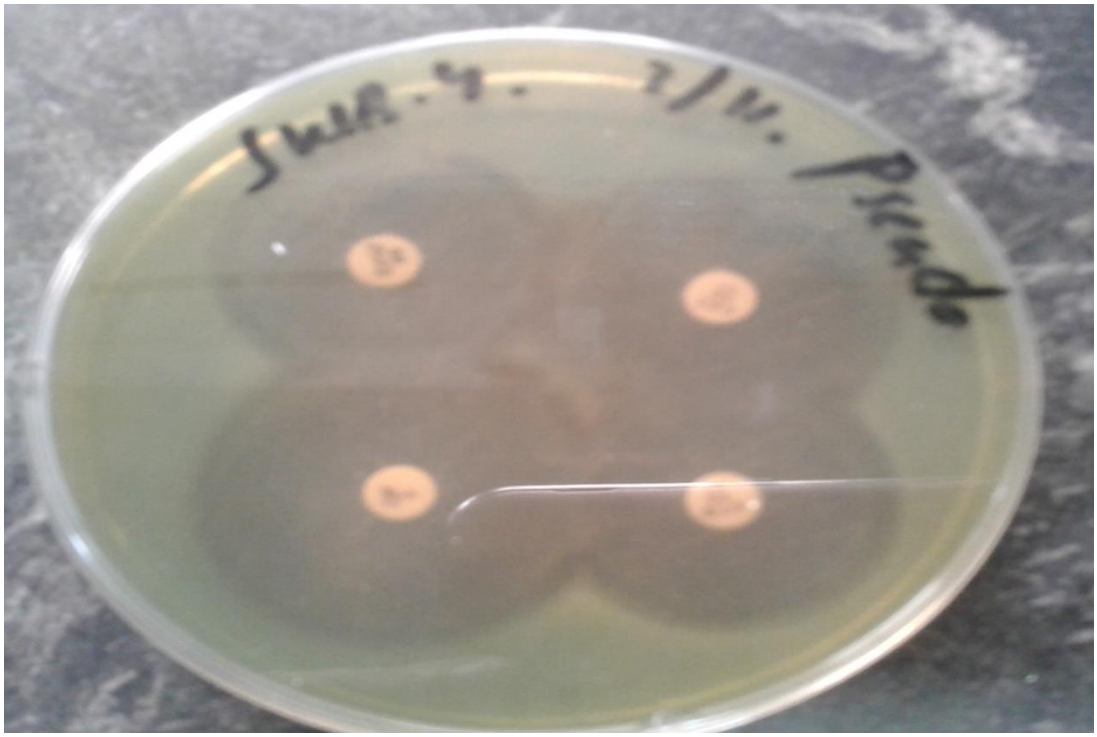


Figure 18 Antibiotic sensitivity test (Sensitive cases)

- **Phenotypic detection of carbapenemase**

Modified Hodge test (MHT)

Then carbapenamase production was checked by the modified Hodge Test. We prepared 0.5 McFarland dilutions of the E-coli (ATCC 25922) in 5 ml of saline. We diluted it to 1:10 by adding 0.5 ml of (0.5 McFarland to 4.5 ml of saline). After this step, we streaked a lawn of the E-coli (ATCC 25922) to Muller Hinton Agar. Control of E coli (ATCC American Type Culture Collection 25922) was obtained from PNS Shifa Hospital Karachi. It dried for 3-5 minutes. Then we kept a 10 µg imipenem susceptibility disc in centre. Then we streaked test organism, positive control and negative control and incubate overnight at 37 ^{o}C for 24 hours. Quality control of the following organisms MHT positive Klebsiella pneumonia ATCC 1705 and MHT negative Klebsiella pneumonia ATCC 1706 were run with each batch of the result. After 24 hours of incubation we examined the plate for a clover –leaf type indentation at the intersection of the test organism E-coli (ATCC 25922) as per Figure 19 and 20 on page 55.

Modified Carbapenem Inactivation Method (mCIM)

We used mCIM method for detection of carbapenemase producing *Pseudomonas aeruginosa*. With the help of sterile inoculating loop, 1µl of test organism was put into 2ml tube of tryptic soy broth (TSB, HiMedia Laboratories) suspension was vortexed for 10-15secs. Then 10µg MEM disk was inserted into 2ml tube as Figure 21 on page 56. Now tube was incubated for 4 hours ± 15 minutes at 35^{o}C±2^{o}C. On the other hand E coli ATCC 25922 with turbidity equivalent to a 0.5 McForland was prepared. Lawning was done on Mueller Hinton Agar. The MEM disk was removed from the TSB suspension with the help of loop and then placed it on Mueller Hinton Agar plate and inoculated for 24 hours. Whenever zones were less than 18mm considered as resistant case as per Figure 22 on page 56.

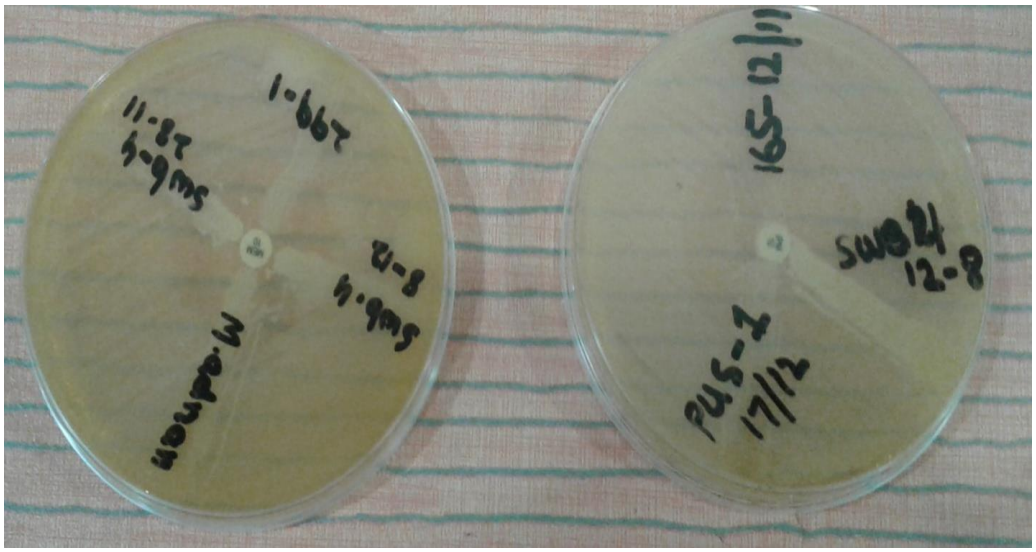


Figure 19 Modified Hodge Test (sensitive case)

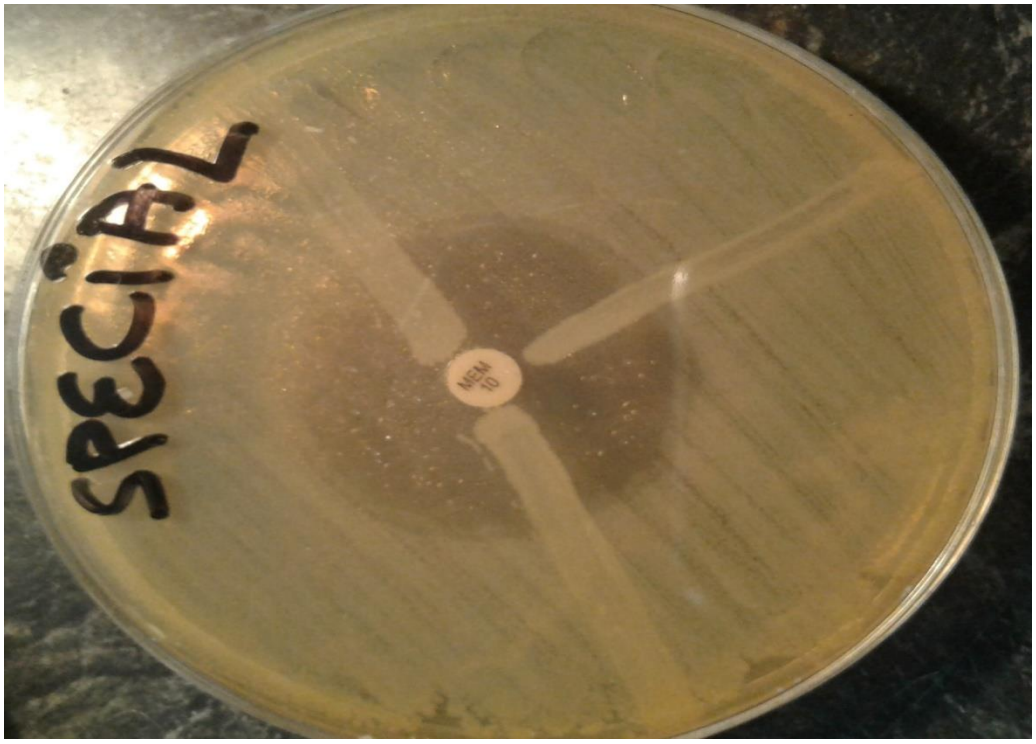


Figure 20 Modified Hodge Method (resistant case)

Figure 21 Inoculation of disc

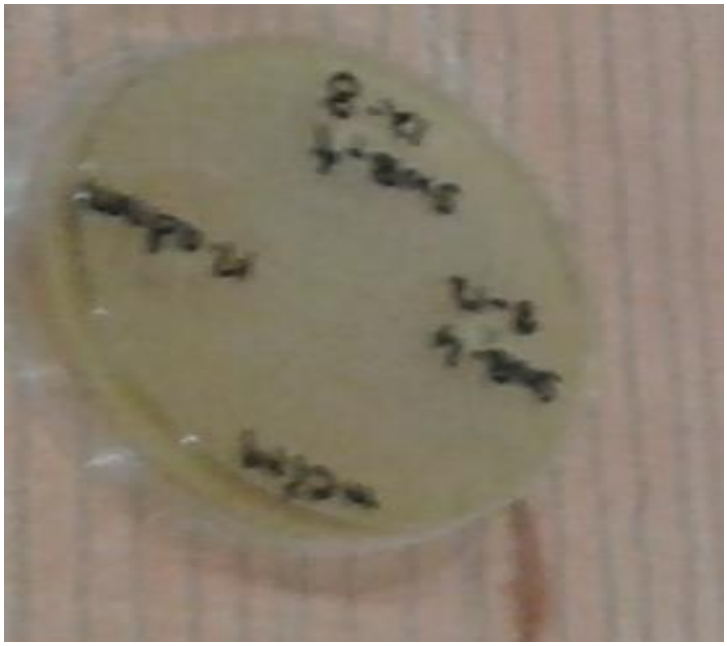


Figure 22 Modified Carbapenem Inactivation Test

GENOTYPIC DETECTION

Detection of OXA-48 genes in cabapenem resistant cases

Protocol:

Pretreatment of Microbial DNA from Cultures (up to $2x10^9$ Bacterial cells)

We added 1.5ml biological fluid into a 2ml tube and centrifuge the tube for 5 min at maximum speed (>14,000Xg). We removed and discarded the supernatant. 400µl Buffer ATL was added and resuspended the pellet (as per Figure 23 on page 58).

40µl Proteinase K was added and mixed the sample by vertexing for 10 seconds. Sample was incubated at 56°C for 10 min. 200 µl Buffer APL2 was added to the sample. Then cap was closed and mixed by pulse-vertexing for 30 seconds. It was incubated at 70°C for 10 min. In order to remove the drops from inside the lid, tube was briefly spinned.

300µL ethanol was added to the lysate. Tube was capped and mixed thoroughly by pulse-vortexing for 15-30 seconds. The lysate was applied carefully into the tube extender of the QIAamp UCP Mini column.

The vacuum pump was switched on. When all lysate had been drawn through the columns completely, switched off the vacuum pump and released the pressure to 0 mbar. Tube extender was removed and discarded carefully.

600µl of Buffer APW1 was applied to the QIAamp UCP Mini column. The lid of the column was left open and switched on the vacuum pump. After all of Buffer APW1 had been drawn through the column, switched off the vacuum pump and released the pressure to 0 mbar.

750µl of Buffer APW2 was applied to the QIAamp UCP Mini column. The lid of the column was left open and switched on the vacuum pump. After all of Buffer APW2 had been drawn through the column, switched off the vacuum pump and released the pressure to 0 mbar. The lid of the QIAamp UCP Mini column was closed and removed it from the vacuum manifold and discarded the VacConnector.

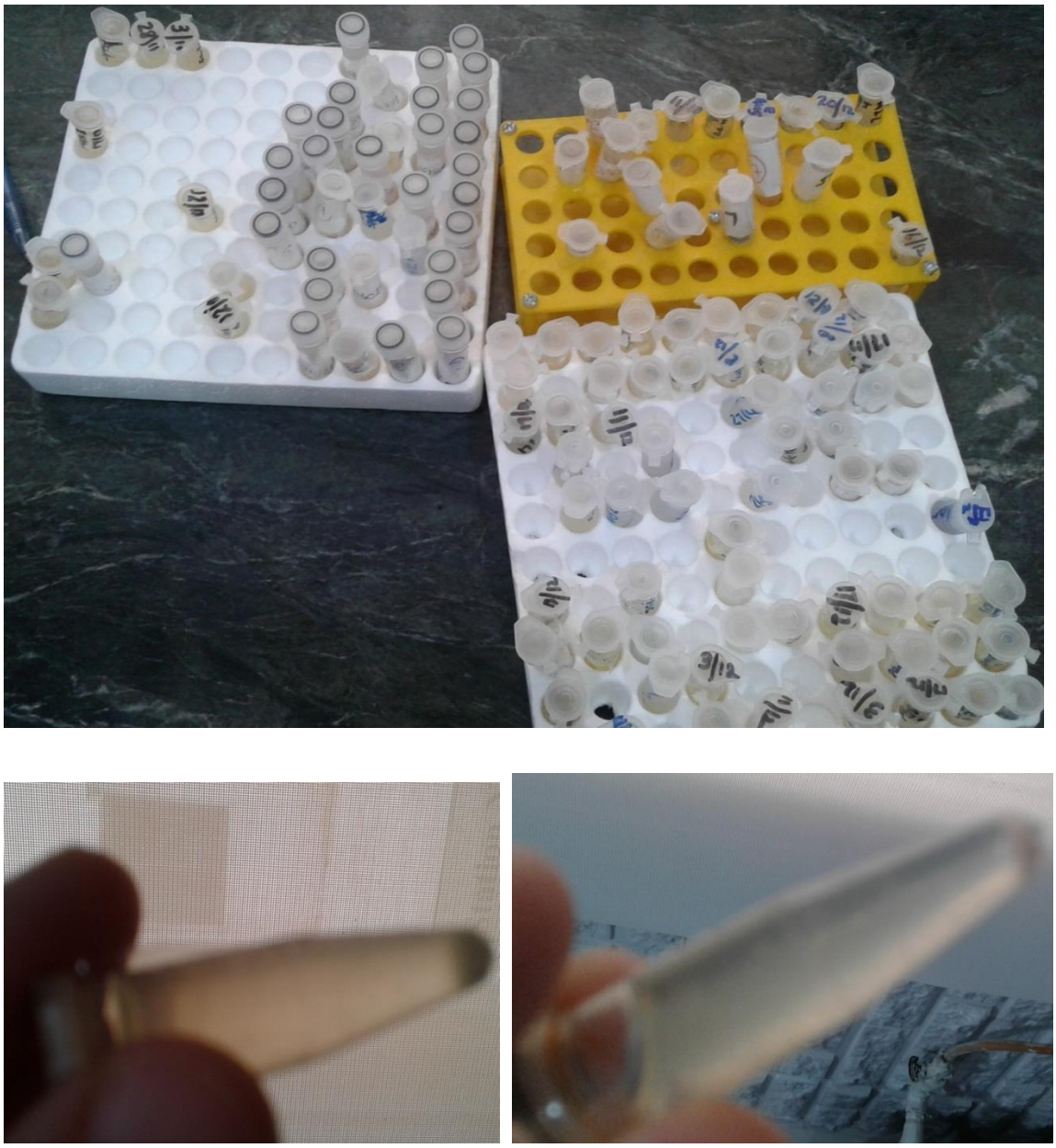


Figure 23 Extracted DNA

Mini column was placed in a clean 2ml collection tube and centrifuged at full speed (20,000xg; 14,000 rpm) for 3 min. Now Mini column was placed into a new 2ml collection tube. Lid was opened and incubated the assembly at 56°C for 3 min to dry the membrane completely.

The Mini column was placed in a clean 1.5ml elution tube and discarded the collection tube. 20-100µl of Buffer AVE was applied carefully to the center of Mini membrane. The lid was closed and incubated at room temperature for 1min. It was centrifuged at full speed (20,000xg; 14,000 rpm) for 1 min to elude the DNA. (Microbial DNA Qpcr Assay/ Multi-Assay Kit catalog no. 330033: BBXX-#######R/F). According to this kit primers were used as per Table 6 on page 60.

Protocol: Real-Time PCR using Microbial DNA Qpcr Assay/ Multi-Assay Kit

According to Table 7 on page 60 reaction mixes were prepared with Microbial DNA positive control (OXA-48), negative Template control and sample.

25µl reaction mixes were dispensed into PCR wells. For Rotor-Gene only, added 20µl reaction mixes per well (as per Table 8 on page 61). Product specification sheet was followed for proper loading sequence for use with data analysis software. PCR plate was tightly sealed with Rotor Disc Heat-Sealing Film.

PCR tubes were placed into the real-time thermal cycler as per Table 9 on page 61. A compression pad was used with the optical Film-sealed plate formats. The run was started. The thermal cycle (C_T) was calculated for each well using the cycler's software. Threshold was taken as 0.02.The resulting threshold cycle values was exported for all wells to a blank Excel spreadsheet for data analysis.

Table 6

Primers for detection of OXA-48 in *Pseudomonas aeruginosa*

Targeted Gene	**Kit Name**	**Primer Name**	**Sequence (5'-3' Direction)**	**Length Bases**	**Amplicon Size, bp**	**Tem in C°**	**Primer conc, pmol/µl**
OXA-48	Qiagen Microbial DNA qPCR Assay Kit	Reverse	ACGACGGCATAGTCATTTGC	20	585 or 597	56	15pmo
		Forward	AACGGGCGAACCAAGCATTTT	21			

Reference (Mlynarcik, Patrik et al., 2016)

Table 7

Reaction List

Reaction no	**Assay**	**Sample**
1	PPC	Sample
2	Microbial DNA qPCR Assay	Microbial DNA-Free Water (NTC)
3	Microbial DNA qPCR Assay	Microbial DNA positive control(OXA-48)
4	Microbial DNA qPCR Assay	Sample

Reference (Qiagen multi-assay kit catalog no 330033)

Table 8

Preparation of reaction mix

Component	Per well(0.2ml PCR style tubes)
Microbial Qpcr Mastermix	12.5µlx12=150µl
Microbial DNA Qpcr Assay(OXA-48)	1µlx12=12
Genomic DNA sample	5ng
Microbial DNA-Free Water	6.5x12=78
Total volume per sample	25µl

Reference (Qiagen multi-assay kit catalog no 330033)

Table 9

Cycling conditions

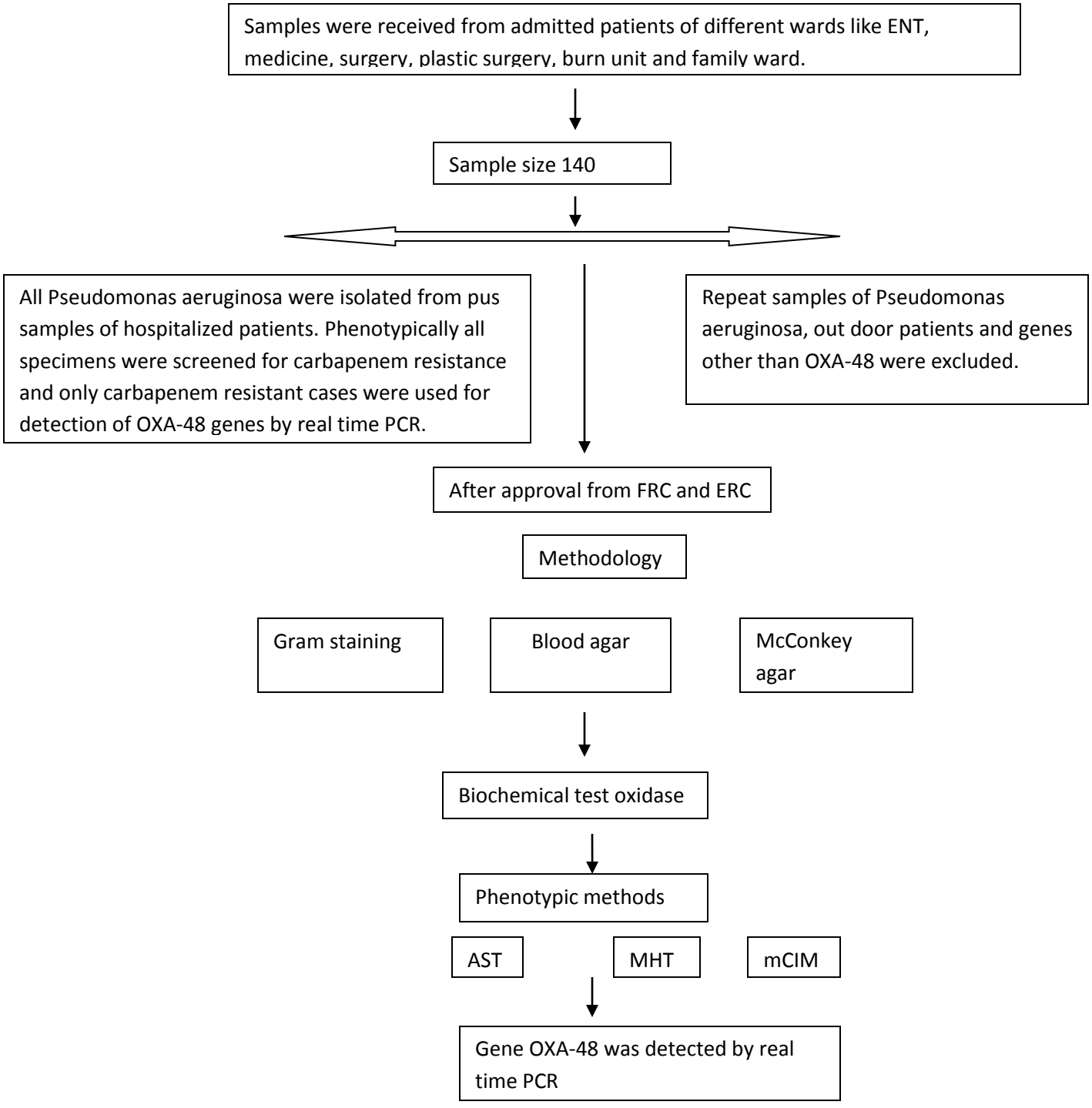
Step	**Time**	**Temperature**	**Number of cycles**
Initial PCR activation step	10min	95°C	1
Step cycling			
Denaturation	15s	95°C	40
Annealin and extension	2min	60°C	40

Reference (Qiagen multi-assay kit catalog no 330033)

3.14 STATISTICAL ANALYSIS

Statistical analysis of the data was done using SPSS version 23.0. Results were reported as frequencies (percentages) for categorical variables i.e source of specimens. Diagnostic accuracy of MHT and mCIM was calculated through sensitivity and specificity after making 2X2 contingency table. Chi square test was applied to see the significance between two categorical variables. AST and PCR were assessed through Chi square test. P-value <0.05 is considered as statistically significant.

3.13 FLOW CHART/ALGORITHM OF STUDY



CHAPTER 4

RESULTS

4.1 Identification and Characterization of *Pseudomonas aeruginosa* isolates:

140 isolates of *Pseudomonas aeruginosa* cultured from pus samples of different infection sites. The most frequent isolates were from ENT infections (47.1%) followed by post-surgical wound infections (13.6%), burns super-infections (12.1%), diabetic foot ulcers (8.6%), plastic post-operative infections (7.9%), pediatric wound infections (1.4%) and officer ward (0.7%) as shown in Table 10 on page 67 and Figure 24 on page 68.

Gram Negative Rods by Gram Staining

By Gram staining *Pseudomonas aeruginosa* appeared as pink rod as per Figure 25 on page 68.

Colonial Appearance on Blood Agar

Pseudomonas aeruginosa clinical isolates yielded colonies which were large, smooth with flat edges, elevated appearance and mostly green and black pigments on blood agar as per Figure 26 on page 69 (Tang YW, 2014).

Differentiation between lactose and non lactose fermenters

MacConkey is a differential media which differentiates between lactose fermenters and non lactose fermenters. *Pseudomonas aeruginosa* is a non lactose fermenter that's why its colonies appeared as coloureless as per Figure 27 on page 69 (Tang YW, 2014).

Biochemical Characterization for *Pseudomonas aeruginosa* Speciation Verification

In order to differentiate *Pseudomonas aeruginosa* from other species of *Pseudomona*s oxidase test was done. *Pseudomonas aeruginosa* colonies turned purple within 10 sec while other species gave purplish colour in more than 10 sec like *Burkhuldaria* as per Figure 28 on page 70.

4.2 Phenotypic Screening Methods

Antibiotic Susceptibility Pattern of Clinical Isolates of *Pseudomonas aeruginosa*

All identified isolates (n=140) of *Pseudomonas aeruginosa* from the clinical specimens were tested for susceptibility against 2 selected antibiotics as per Table 11 and Figure 29 on page 71 & Figures 30, 31 on page 72.

Among the antibiotics tested were the selected "anti-*Pseudomonal*" drugs especially cabapenems like meropenem (MEM) (Oxoid) and imipenem (IMP) (Oxoid).

The resistance and susceptibility profile to both antibiotics were tested by the Kerby-Bauer disk diffusion method consistent to the protocol of the Clinical Laboratory and Standards Institute (CLSI), USA. Out of 140 *Pseudomonas aeruginosa* isolates, 17 were found to be resistant to carbapenem (Meropenem and Imipenem) drugs.

Modified Hodge Test (MHT)

The results of MHT showed 20 resistant cases with 77% sensitivity, 99.1% specificity, 95.2% positive predictive value and 100% negative predictive value as per Table 12 and Figure 32 on page 73, Figures 33 and 34 on page 74.

Modified Carbapenem Inactivation Method (mCIM)

The results of mCIM showed 25 cases out of 140 as resistant with 100% sensitivity, 99.1% specificity, 96.1% positive predictive value and 100% negative predictive value as per Table 13 and Figure 35 on page 75.

Comparison of three Phenotypic Methods

Carbapenemase detection was done more efficiently with mCIM as compare to MHT and AST as per Pie chart Figure 36 on page 76.

Comparison of MHT and mCIM

Both tests were compared with help of chi square and p-value was significant as 0.000 as per Table 14 on page no 77.

4.3 Genotypic detection for confirmation of OXA-48 Gene

Real-Time Polymerase Chain reaction, also known as quantitative polymerase chain reaction, is a laboratory technique of molecular biology based on the polymerase chain reaction. It monitors the amplification of a targeted DNA molecule during the PCR i.e in real time.

Confirmation of extracted DNA

Extracted DNA was confirmed from Molecular Laboratory of Dow University of Health Sciences as per Table 15 on page 77.

Quantitative information

OXA-48 gene was investigated in beta-lactamase producing *Pseudomonas aeruginosa* by using real-time PCR. 4 cases out of 25 carbapenem resistant isolates (n=4) (16%) exhibited OXA-48 gene as per Figure 39 on page 86 and Table 19 on page 87.

Table 10

Unit Wise Distribution of *Pseudomonas aeruginosa* in PNS Shifa

Ward	Frequency	Percent (%)
ENT*	66	47.1
SURGERY	19	13.6
BURN UNIT	17	12.1
10-ICU*	12	8.6
MEDICINE	12	8.6
PLASTIC SURGERY	11	7.9
PAEDRIATICS	2	1.4
OFFICER WARD	1	.7

*ENT Ear, Nose and Throat

*ICU Intensive Care Unit

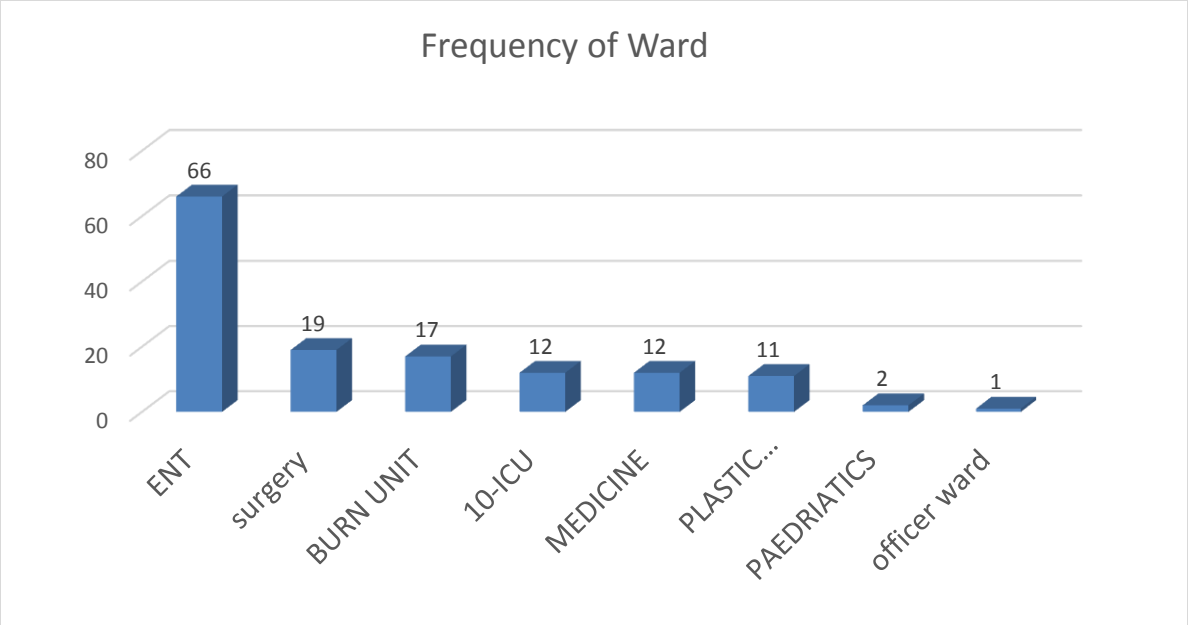


Figure 24 Graph 1 Graphical presentation of frequency of wards

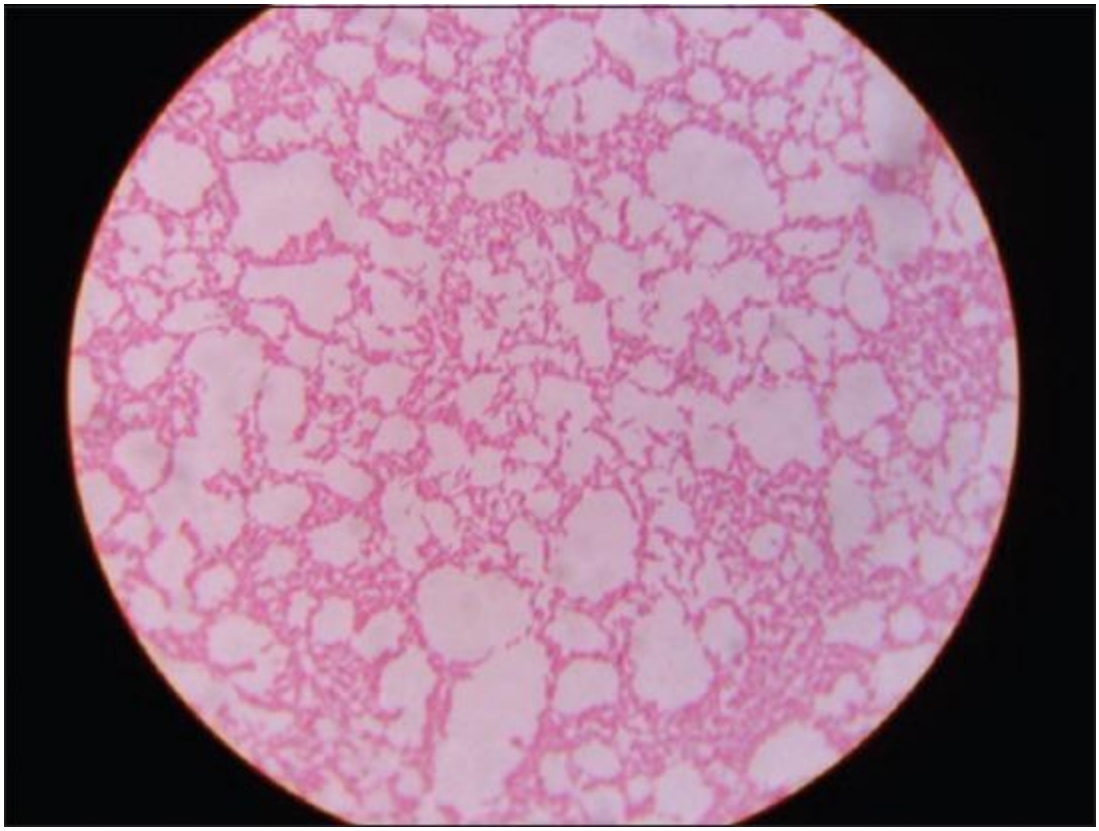


Figure 25 Gram negative rods *Pseudomonas aeruginosa* (Banerjee et al., 2017)

Figure 26 Colonies of *Pseudomonas aeruginosa* on blood agar

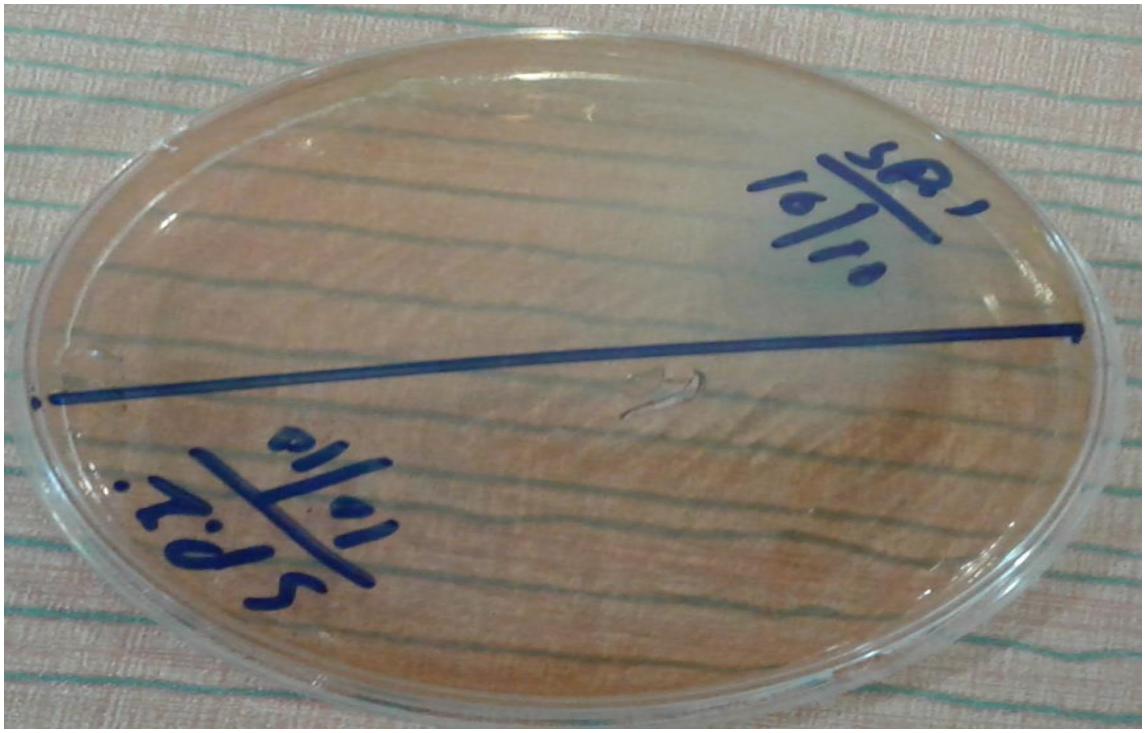


Figure 27 Colonies of *Pseudomonas aeruginosa* on MacConkey agar

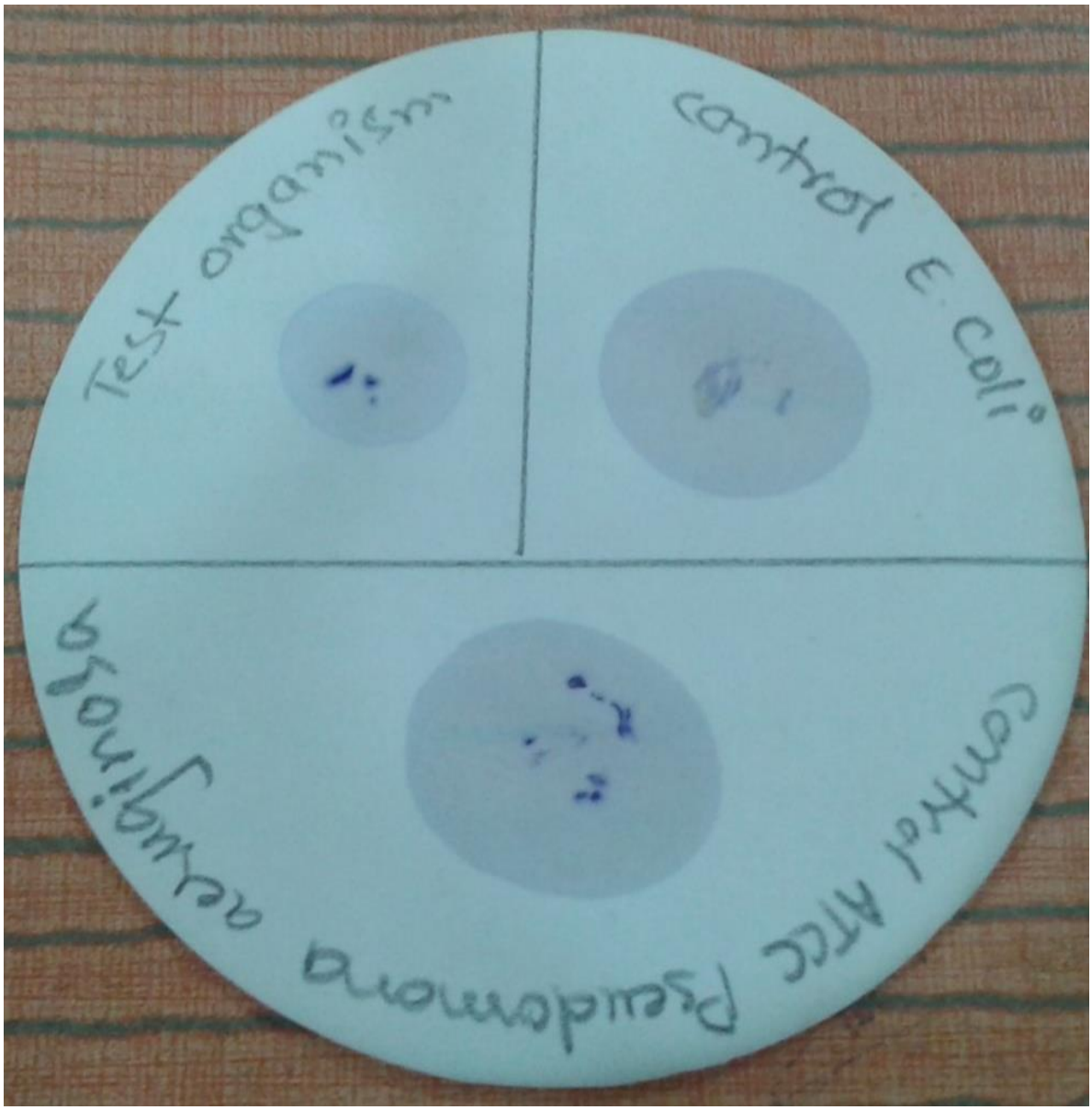


Figure 28 Oxidase test

Table 11

Antibiotic Resistance Profile of Carbapenem from clinical isolates

(n=140)

Carbapenems	R<=15 mm	I 16-18mm	S >=19 mm
IMP	17 12%	0 0%	123 87%
MEM	17 12%	0 0%	123 87%

R Resistant IMP Imipenem MEM Meropenem

I Intermediate

S Sensitive

Figure 29 Antibiotic (Carbapenem) susceptibility test

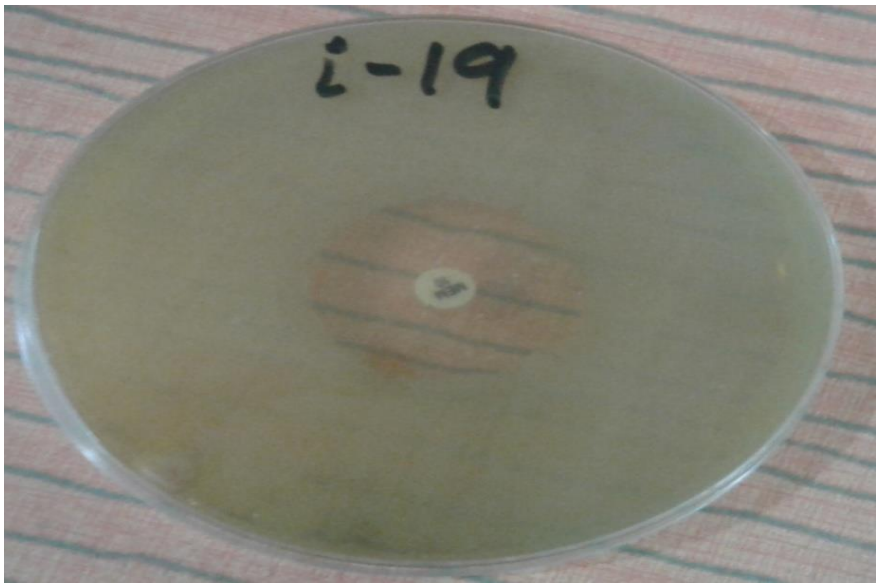


Figure 30 Antibiotic (Carbapenem) susceptible zones

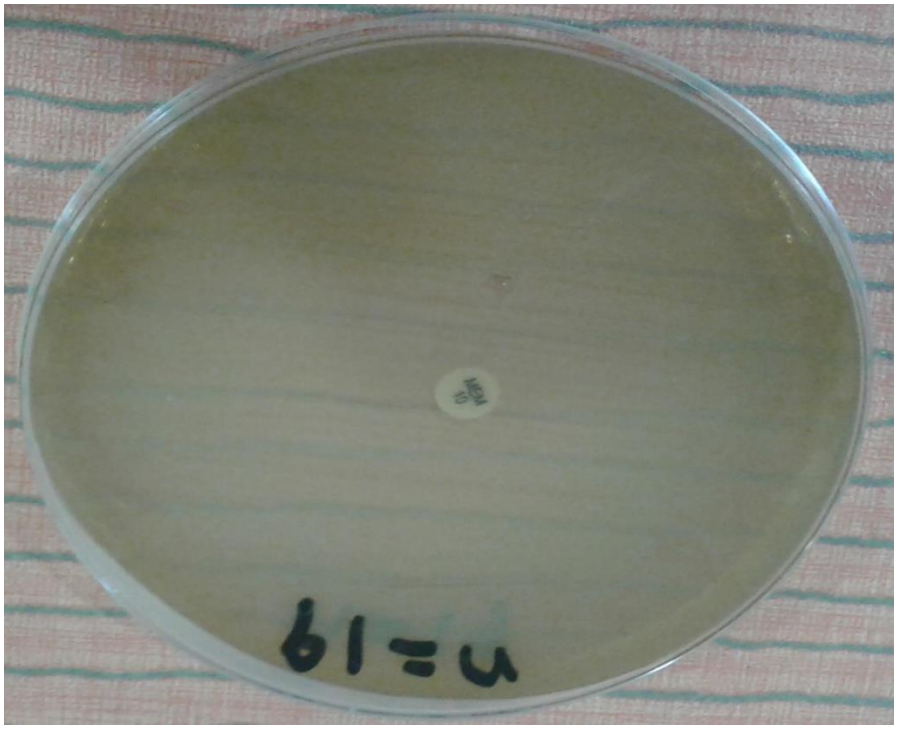


Figure 31 Antibiotic (Carbapenem) Resistance Zone

Table 12

2x2 contingency Table for MHT

	MHT		
mCIM	POSITIVE	NEGATIVE	Total
POSITIVE	20	1	21
NEGATIVE	6	113	119
Total	26	114	140

MHT Modified Hodge Test

mCIM Modified carbapenem inactivation method

Sensitivity 77%, Specificity 99.1%, Positive predictive value 95.2%, Negative predictive value 100%

Figure 32 Modified Hodge Test

Figure 33 MHT without indentation (negative)

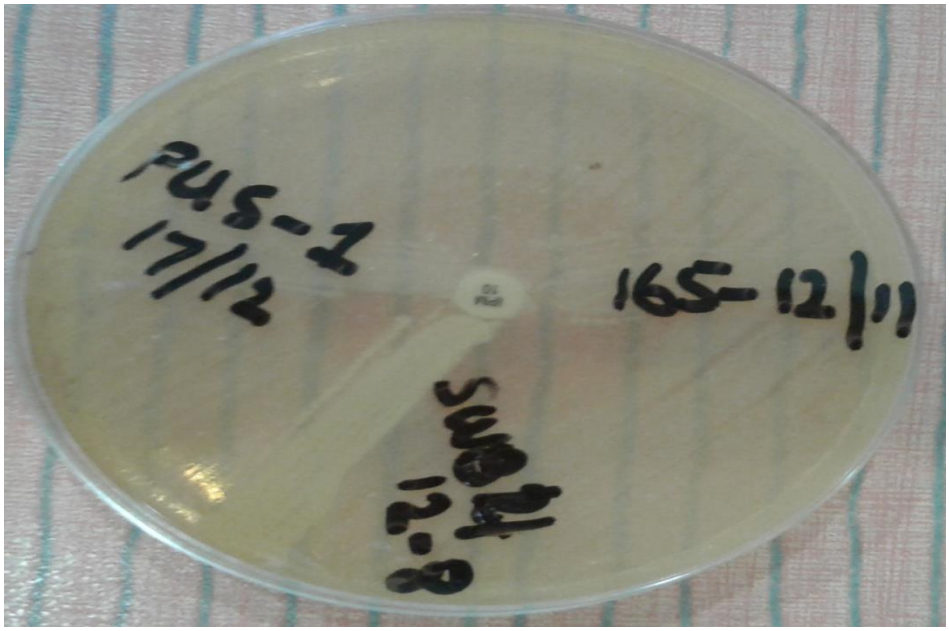


Figure 34 MHT with indentation (positive)

Table 13

2x2 contingency Table for mCIM

	Mcim		
MHT	POSITIVE	NEGATIVE	Total
POSITIVE	25	1	26
NEGATIVE	0	114	114
Total	25	115	140

mCIM Modified Carbapenem inactivation Method

MHT Modified Hodge Test

Sensitivity 100%, Specificity 99.1%, Positive predictive value 96.1%, Negative predictive value 100%

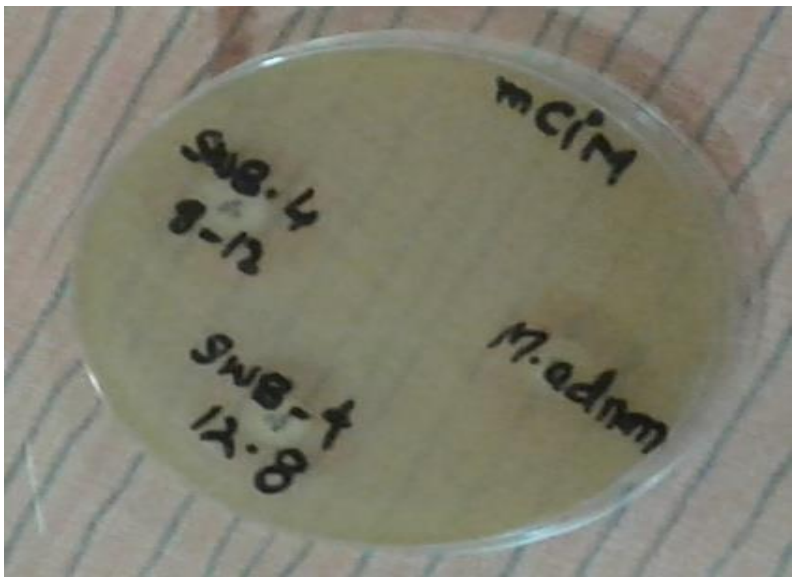


Figure 35 Modified Carbapenem Inactivation Method

FIGURE 36 COMPARISON OF THREE PHENOTYPIC METHODS

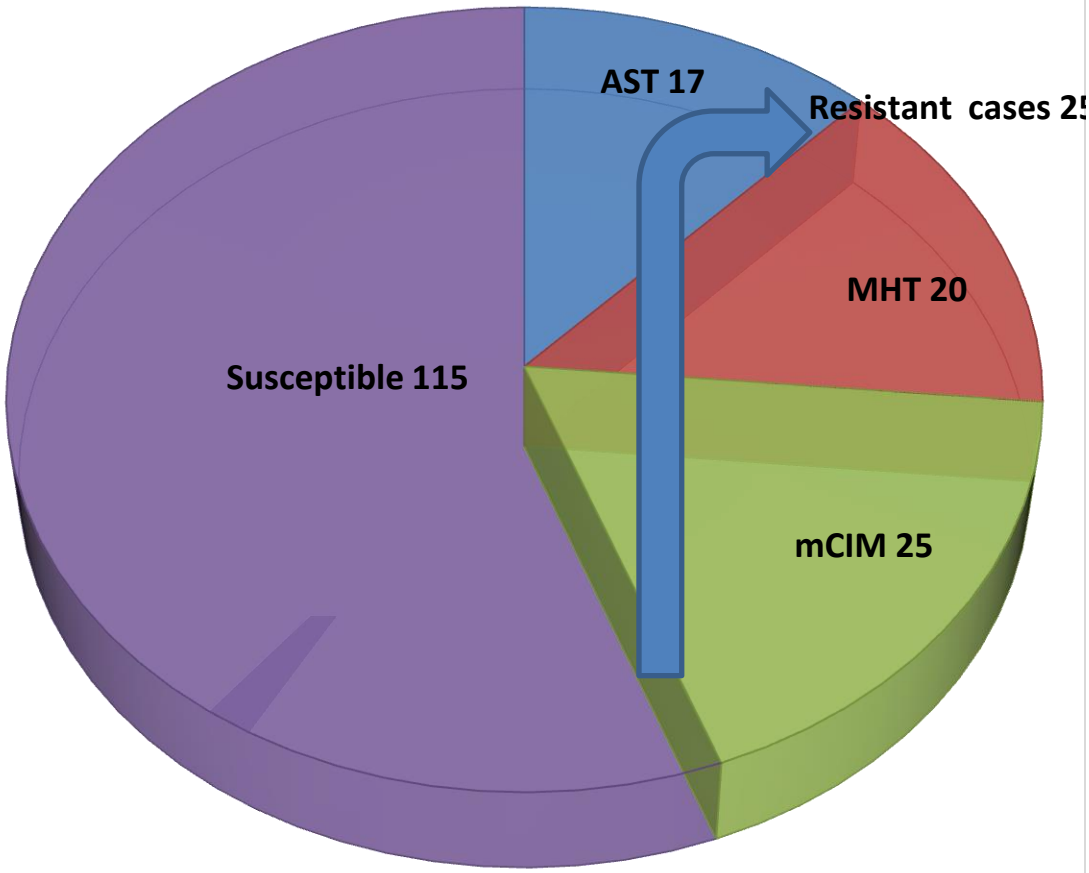


Table 14

Comparison of MHT and mCIM

	mCIM		MHT		P-value
	Frequency	**Percent**	**Frequency**	**Percent**	
Positive	25	17.9	20	14.29	0.000
Negative	115	82.1	120	85.71	
Total	140	100.0	140	100	

mCIM Modified Hodge Test MHT Modified Hodge Test

Statistically significant*

Table 15

Confirmation of extracted DNA

Samples	Yield
1	**54.90g/µl**
2	**45.81g/µl**
3	**93.66g/µl**
4	**67.30g/µl**
5	**89.43g/µl**
6	**185.70g/µl**
7	**288.67g/µl**
8	**82.10g/µl**

µl micro liter g gram

Quantitation Report

Experiment Information

Run Name	Dr shaista Bakhat OXA- 48 2019-01-23 (1)
Run Start	1/23/2019 10:46:27 AM
Run Finish	1/23/2019 1:16:45 PM
Operator	Dr Shaista Bakhat
Notes	OXA- 48
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	1.33
Gain Yellow	9.33
Gain Orange	8.
Gain Red	5.
Gain Crimson	7.
Machine Serial No.	0216153

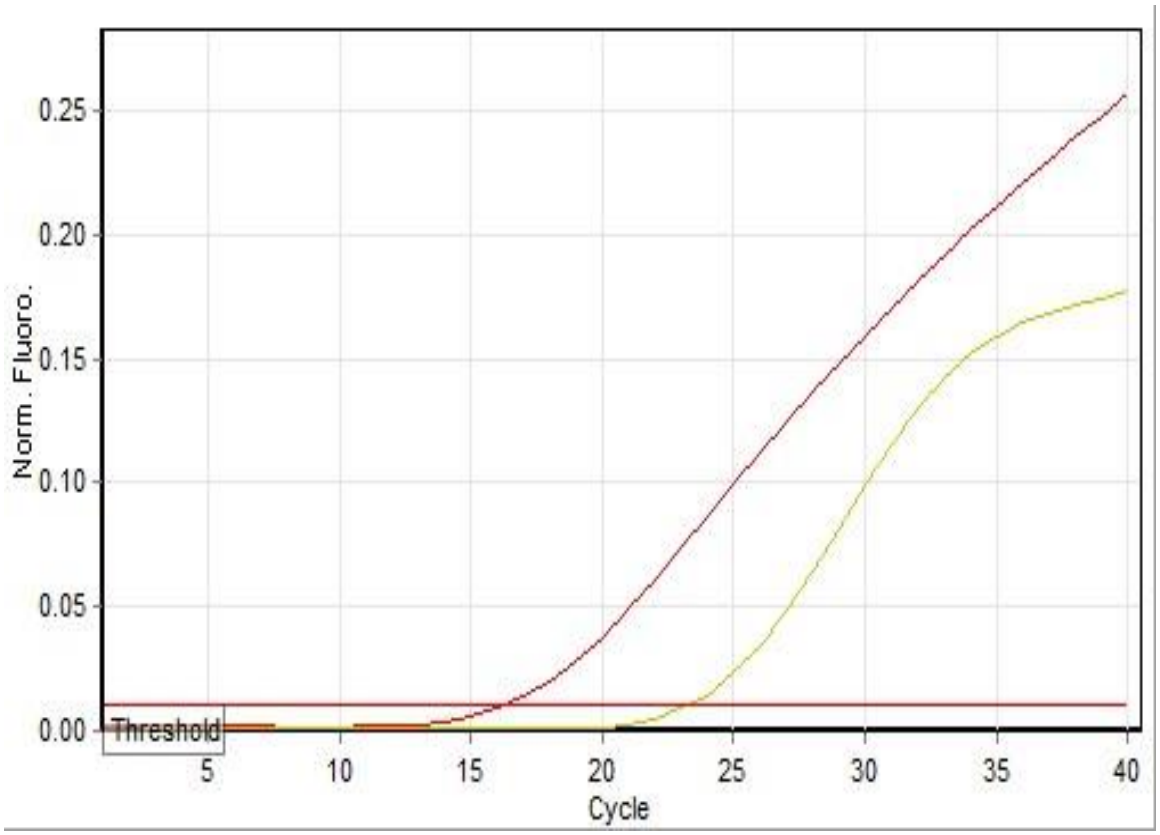


Figure 37 OXA-48 Negative cases (First cycle)

Table 16

OXA-48 negative cases (First cycle)

No.	Color	Name	Type	Ct	Ct Comment
1		PPC	Positive Control	16.51	
2		Postive control	Positive Control	23.43	
3		Negative control	NTC		NEG (NTC)
4		1	Unknown		NEG (NTC)
5		2	Unknown		NEG (NTC)
6		3	Unknown		NEG (NTC)
7		4	Unknown		NEG (NTC)
8		5	Unknown		NEG (NTC)
9		6	Unknown		NEG (NTC)
10		7	Unknown		NEG (NTC)
11		8	Unknown		NEG (NTC)
12		Negative control	Negative Control		NEG (NTC)

NEG Negative

Quantitation Report

Table 17

OXA-48 negative cases (second cycle)

PPC	Positive Control	16.51	
Postive control	Positive Control	23.43	
Negative control	NTC		NEG (NTC)
9	Unknown		NEG (NTC)
10	Unknown		NEG (NTC)
11	Unknown		NEG (NTC)
12	Unknown		NEG (NTC)
13	Unknown		NEG (NTC)
14	Unknown		NEG (NTC)
15	Unknown		NEG (NTC)
16	Unknown		NEG (NTC)
Ngative control	Negative Control		NEG (NTC)

NEG Negative

Experiment Information

Run Name	Dr shaista Bakhat OXA- 48 2019-02-04 (1)
Run Start	04/02/2019 12:57:16 PM
Run Finish	04/02/2019 3:08:38 PM
Operator	Dr Shaista Bakhat
Notes	OXA- 48
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	4.
Machine Serial No.	0216153

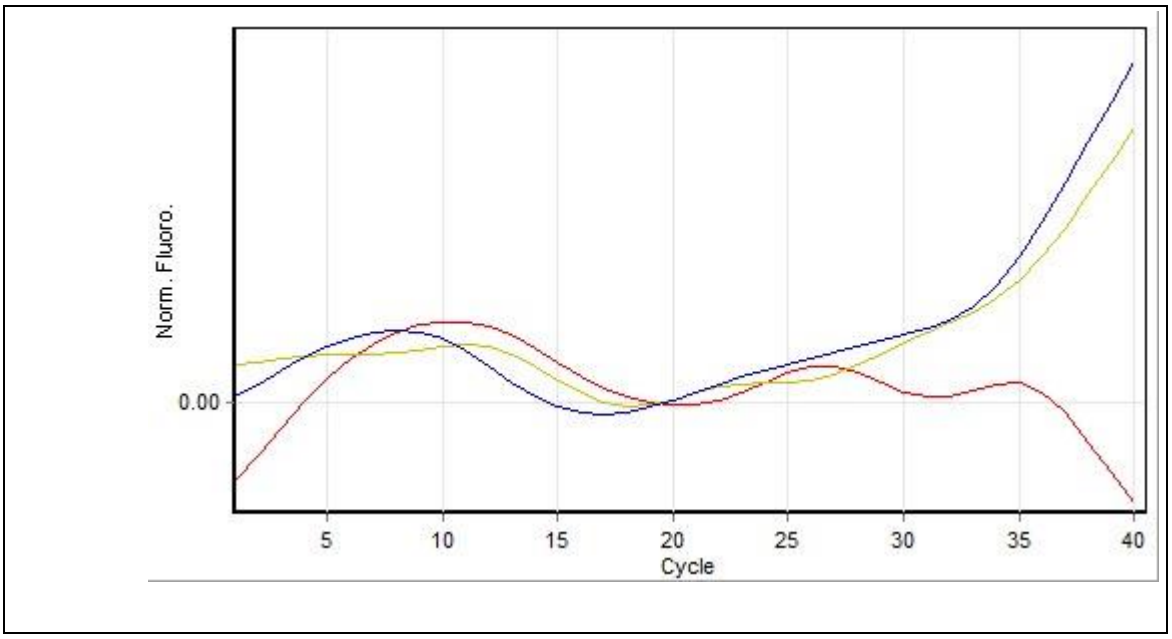


Figure 38 OXA-48 negative cases (second cycle)

Table 18

OXA-48 negative cases (3rd cycle)

No.	Color	Name	Type	Ct
17		4c—10	Unknown	Neg
18		pseudo 212	Unknown	Neg
19		swab –5	Unknown	Neg
20		Pus sample	Unknown	Neg
21		Pus sample	Unknown	Neg

Neg Negative

Quantitation Report

Experiment Information

Run Name	Dr Shaista OXA- 48 2019-01-29 (1) rex 2
Run Start	29/01/2019 12:52:04 PM
Run Finish	29/01/2019 3:21:31 PM
Operator	Dr Shaista Bakhat
Notes	OXA- 48
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	4.
Gain Yellow	9.33
Gain Orange	8.
Gain Red	5.
Gain Crimson	7.
Machine Serial No.	0216153

OXA 48 Carbapenemase gene

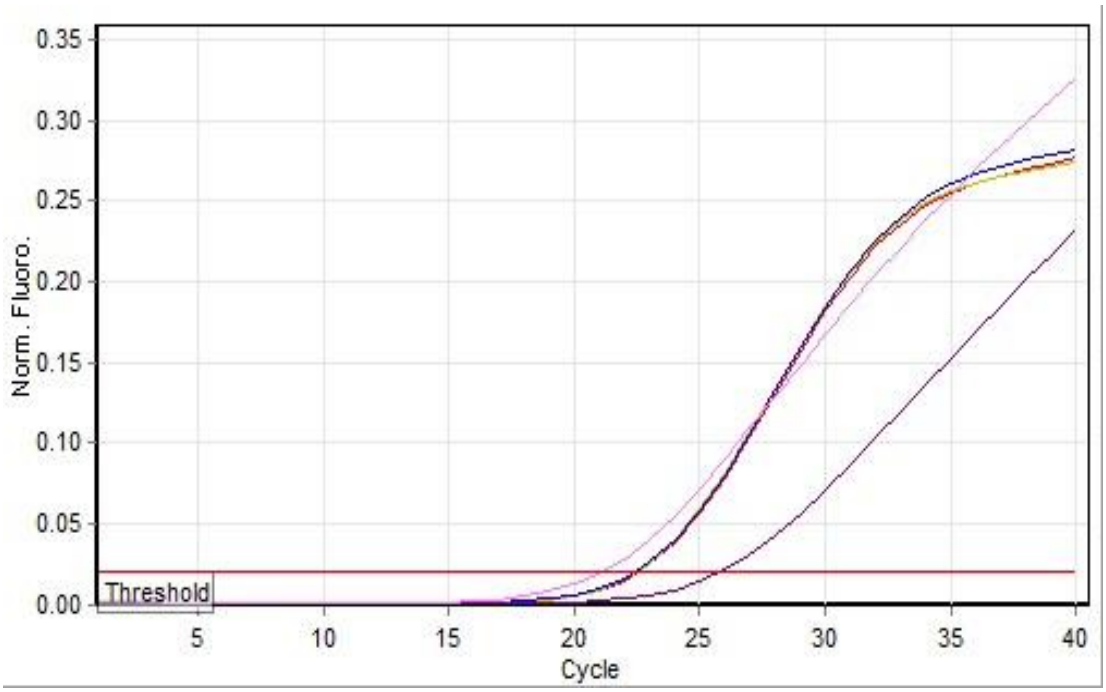


Figure 39 Real Time PCR (3rd cycle)

Table 19

OXA-48 positive cases (fourth cycle)

No.	Color	Name	Type	Ct	Ct Comment
1		Positive control	Unknown	22.58	
22		sample 1	Unknown	22.51	Positive
23		sample 2	Unknown	22.45	Positive
24		sample 3	Unknown	25.79	Positive
25		sample 4	Unknown	21.13	Positive

Run Name	Dr. Shaista OXA-48 2019-01-23 (1)
Run Start	1/23/2019 10:46:27 AM
Run Finish	1/23/2019 1:16:45 PM
Operator	Dr. Shaista Bakhat
Notes	OXA-48
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	1.33
Gain Yellow	9.33
Gain Orange	8.
Gain Red	5.
Gain Crimson	7.

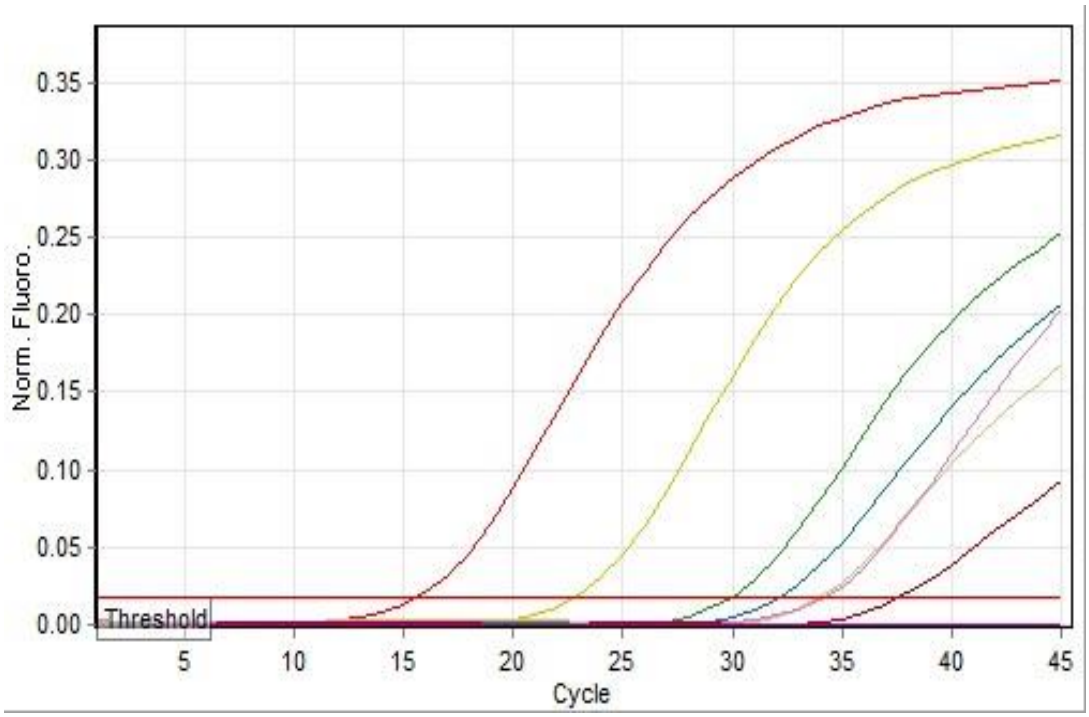


Figure 40 Cumulative result OXA-48 positive cases

Table 20

OXA-48 Cases (cumulative report)

No.	Color	Name	Type	Ct	Ct Comment
1		PPC	Positive Control	15.75	
2		Positive Control	Positive Control	22.86	
		Sample 1	Unknown		NEG (NTC)
		Sample 2	Unknown		NEG (NTC)
		Sample 3	Unknown	32.24	Positive
		Sample 4	Unknown		NEG (NTC)
		Sample 5	Unknown	29.95	Positive
		Sample 6	Unknown		NEG (NTC)
		Sample 7	Unknown		NEG (NTC)
		Sample 8	Unknown		NEG (NTC)
		Sample 9	Unknown	34.08	Positive
		Sample 10	Unknown		NEG (NTC)
		Sample 11	Unknown		NEG (NTC)
		Sample 12	Unknown	34.27	Positive
		Sample 13	Unknown	37.68	NEG

No.	Color	Name	Type	Ct	Ct Comment
					(NTC)
		Sample 14	Unknown		NEG (NTC)
		Sample 15	Unknown		NEG (NTC)
		Sample 16	Unknown		NEG (NTC)
		Sample 17	Unknown		NEG (NTC)
		Negative Control	Negative Control		NEG (NTC)
		Sample 18	Unknown		NEG (NTC)
		Sample 19	Unknown		NEG (NTC)
		Sample 20	Unknown		NEG (NTC)
		Sample 21	Unknown		NEG (NTC)
		Sample 22	Unknown		NEG (NTC)
		Sample 23	Unknown		NEG (NTC)
		Sample 24	Unknown		NEG (NTC)
		Sample 25	Unknown		NEG (NTC)

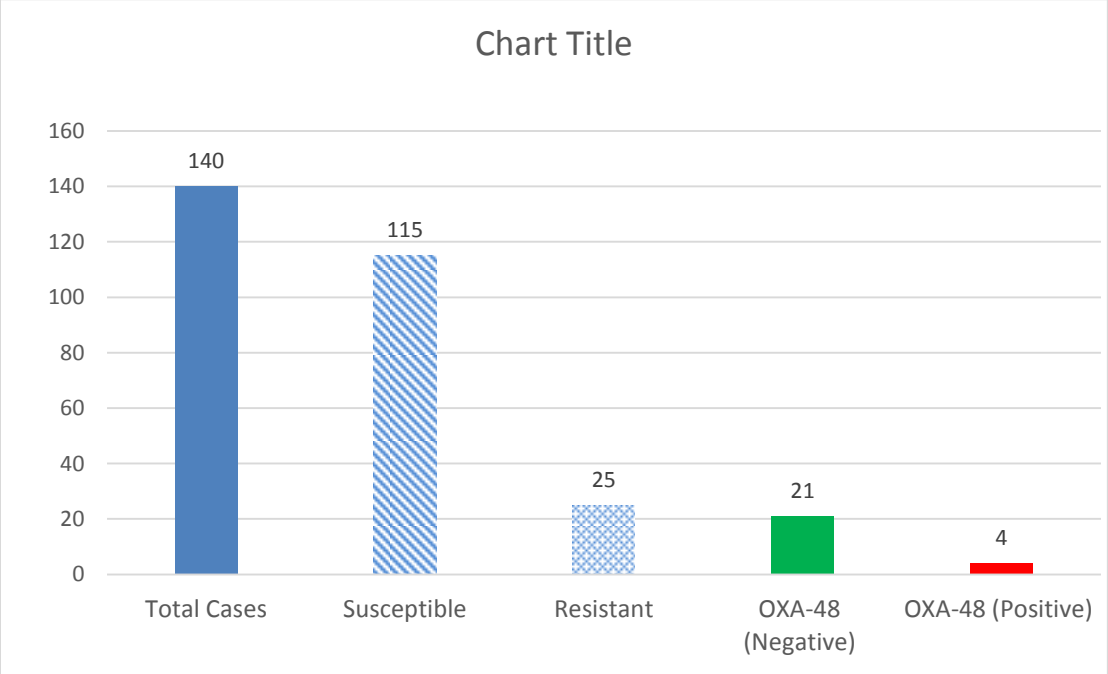


Figure 41 OXA-48

CHAPTER 5

DISCUSSION

High risk of hospital-acquired infections over the last ten years has inculcated a fear among health-care facilitators and communities (Judd et al., 2016). Center for disease control (CDC) stated that *Pseudomonas aeruginosa* is responsible for 10.1% of all nosocomial infections in the United States.

Sheikh et al., (2008) showed 29.1% nosocomial infections were caused by *Pseudomonas aeruginosa* in Pakistan. While, the public's imagination has been captivated by the possibility of a devastating epidemics of influenza virus, dengue fever, MRSA and VRSA, infections by carbpenem resistant Gram negative rods particularly *Pseudomonas aeruginosa*, insidiously raging and has already made its stamp all over the world (Meletis, 2016).

According to record, the prevalence of extended spectrum beta lactamase is high in Pakistan, that is, above 80% (Nahid et al., 2013). Carbapenems (imipenem, meropenem, ertapenem and doripenem) have a broad antimicrobial range; these are last resort for controlling hospital acquired infections especially among Gram negative rods. However, in recent times, carbapenemase-producing micro-organisms that block the effects of these antibiotics have increased all over the world (Patel and Bonomo, 2013).

Carbapenem resistance of Gram negative organisms is a constant health problem of global dimensions. Carbapenemase producing organisms (CPO) cause diverse types of hospital acquired infections like ventilator acquired pneumonia or urine infections which are physician's nightmare to treat. Infections caused by hospital acquired CPOs have very high mortality rate up to 70% because of failure of therapy regimes. This type of drug resistance particularly when mediated by transferable genes between susceptible and resistant bacterial population is spreading fast. It is responsible for serious epidemics and reducing therapy options for physicians. Therefore, there are many laboratory procedures for screening of carbapenem resistant *P.aeruginosa*; some screening methods for detection of CRPA are Modified Hodge test and modified Carbapenem inactivation methods (Doyle et al., 2012).

The results of phenotypic and genotypic methods for detecting carbapenem were tested for 140 *Pseudomonas aeruginosa* strains isolated from pus samples in PNS Shifa during one-year period (2018-19). Sample size was calculated with prevalence 10.2%, 95% confidence interval and 5% margin of error. It was also noted *P. aeruginosa* were detected more in summer season. We got 17 carbapenem resistant cases by antimicrobial susceptibility testing followed by Modified Hodge Test; we got 20 carbapenem resistant cases and 25 by mCIM. 12% carbapenem resistance was indicated by AST method, 14.2% by MHT and 17.8% by mCIM.

(Yin et al., 2018; Sousa et al., 2017) illustrated that *P.aeruginosa* is the major cause of infection in burn patients in contrast to our study that showed high frequency rate in ENT(Ear, Nose and Throat) ward.

Ansari, Salman and Yaqoob (2015) identified high rate of *Pseudomonal* infections in surgical ward followed by pediatric ward.

Banjare and Barapatre, (2015) described the prevalence of carbapenem-resistant *P. aeruginosa* in India. 10.46% carbapenem resistant cases were detected in *P. aeruginosa* which is in association with current study.

The percentage of the *Pseudomonas aeruginosa* isolates expressing resistance to carbapenem by AST method was higher in our study as compare with a previous study which showed (9.3%) resistance conducted by (Abbas et al, 2015). Shah, Wasim, and Abdullah (2015) found no resistance for imipenem.

While Lopez-Gracia et al., (2018) detected that carbapenems are 68% resistant towards *P aeruginosa* by AST methods in Mexican Hospital.

Wi et al., (2017) in Republic of Korea detected 23.4% resistance carbapenem by AST method.

On the other hand Samad A et al., (2017) detected low level of resistance 8.45% of carbapenem resistance towards *P. aeruginosa.*

In our study, we also checked for carbapenemase enzyme which mediates resistance towards carbapenem (meropenem and imipenem) by the Modified Hodge Test and mCIM. The MHT and mCIM were carried out at 35°C±2°C for 24 hours. According to our study, MHT showed 77% sensitivity and mCIM exhibited 99.1% sensitivity. Our results indicated that the mCIM had high sensitivity for carbapenemase detection unlike the MHT. Although the mCIM test requires a longer incubation time, it provides excellent sensitivity.

The results of our study are in agreement with the findings of multiple studies. These studies showed carbapenem resistance by MHT (Modified Hodge Test) and mCIM (Modified Carbapenem Inactivation Method) and these studies validated mCIM as effective for screening of carbapenem resistant organisms. They recommended mCIM to be introduced in laboratories for identification of carbapenem resistance in *Pseudomonas aeruginosa* isolates. They concluded mCIM is a simple and inexpensive test for detection and prevention of carbapenem resistant strains (Pierce et al., 2017; Sfeir, Faunteroy and Jenkins, 2018; Akhi et al., 2016; Pawar et al., 2018; Farooqui F, 2017).

The results of our study are in accordance with the findings of various researches. These studies detected carbapenem resistance in cases of hospitalized patients by MHT and mCIM. These studies showed less sensitivity and specificity of MHT as compare to mCIM. MHT gave false negative results for carbapenemase OXA-48 producers. It is not an accurate method and can not be used in laboratory for detection of carbapenemase (Song, 2016; Kuchibiro et al., 2018; Yamada et al., 2016; Khalili et al., 2016; McMullen et al., 2017; Van der Zwaluw et al., 2015; Lisbo et al., 2018).

Therefore we conclude that mCIM as a phenotypic method for detection of carbapenemase production in *Pseudomonas aeruginosa* isolates can be recommended as gold standard method.

According to their amino acid sequences and inhibitor profile, they are divided into four sub classes(A, C, D) and fourth one is (B) that contains zinc at active side (Hall & Barlow, 2005). Among these classes C is insignificant. They have different enzymes like Group A carbapenemases (KPC and GES enzymes), Group B metallo-β lactamases (IMP,

VIM and NDM β-lactamases) and Group D carbapenemases (OXA23,24/40, 48, 51, 55, 143) have recently emerged(187, 189, 242, 243, 253).

During the last decade, CPOs (carbapenem producing organisms) which are resistant to all the commonly prescribing antibiotics have come forward. CPOs producing genes NDM-1 carbapenemase are a danger, as this gene is vastly mobile, being readily communicable in between same species and genera with simultaneous transmit up to 14 different antibiotic resistance genes. There is substantial proof that CPOs with NDM-1 is present in the India & Pakistan and that they are disseminating through international travel.

On the whole prevalence rate of 18.5% *bla*NDM-1 positive *Enterobacteriaceae* has been found in 2011 from two military hospitals in Rawalpindi, Pakistan (Perry et al., 2011). A second study performed in two tertiary care hospitals in Pakistan from 2013 specified prevalence rates of 23.0% for *bla*NDM-1, 25.0% for *bla*VIM and 1.5% for *bla*IMP (Nahid et al., 2013).

Since *bla*NDM-1 was first identified in India in 2009, it is supposed that CPOs is vastly disseminated globally from this area (Kumarasamy et al., 2009).

The recent global emergence of carbapenem resistance in Gram-negative organisms poses an alarming public threat. Periodic outbreaks of carbapenem-resistant organisms have been identified not only in hospitals, but also in the community more so in the summer season as we found in our study.

While *bla*KPC is the most reported carbapenemase gene globally, *bla*NDM is demonstrated to be the most prevalent gene in our part of the world. One study at a military hospital informed that *bla*NDM was the only carbapenemase resistance gene found in all samples (Day et al., 2013).

Kazi M et al., (2018) identified carbapenem resistant group by genotypic analysis. This study found 83.3% were carbapenemases producers of which 24.3% were OXA-48 producers by real time PCR.

Mohanty S et al., 2017 identified through their study the presence of carbapenemase-mediated resistance. Carbapenemase-encoding genes were amplified by PCR. The frequency of carbapenemase genes oxacillinas (OXA)-48 was 24.7%. This is high frequency rate as compare to Pakistan.

Qureshi et al., (2018) stated 11% bla_{VIM} producing *P. aeruginosa* in Pakistan.
Khan et al., (2016) stated that bla NDM-1 gene-positive *Klebsiella pneumonia* isolates were detected from samples of (CCU) and 65% NDM-1 strains were detected from patients at extreme ages. Sattar et al., (2014) detected KPC-2 and bla_{NDM-1} co-existence in isolates of *Klebsiella pneumonia* in Pakistan.

Hsu et al., (2017) described that OXA-23 was the most common carbapenemase in Pakistan, which was detected in *Acinetobacter baumannii.* From 2002-2007, 0.1% carbapenem resistance was present in *Klebsiella pneumoniae.* Aga khan university showed 1%-3% carbapenem resistance in *E coli* and *Klebsiella pneumonia* in 2009 which was followed by 5% and 18% by 2014. In 2010, CP-CRE (carbapenemase-producing Enterobacteriaceae) rate was 18.5%. OXA-48 positive *P. aeruginosa* have not been found in Pakistan to date.

In our study we only look for bla OXA 48 as it has not been reported in previous study in Pakistan, although it is already reported in other parts like India, Afghanistan, Turkey and Bangladesh.
In our study we found OXA-48 gene by Real time PCR was 16% only in hospitalized patients of PNS Shifa. Real Time-PCR is the amplification of DNA. It is measured in real time by using fluorescent reporter. The fluorescent reporter signal strength is proportional to amplified DNA.

Bonnin et al., (2018) reported OXA-48 producing *P.aeruginosa* strains in India.

Abdalhamid et al., (2016) discovered carbapenemase genes like TEM, SHV, OXA-48, IMP, KPC, SIM, SPM and GIM from isolates of *P.aeruginosa* and *Enterobacteriacea*e in Saudi Arabia.

Duin and Doi, (2017) also reported the carbapenem resistance in Turkey, where the rate of carbapenem resistance is very high. The 92% of carbapenem resistant *Enterobacteriaceae* were harboring OXA-48 genes. Spread of OXA-48 is widespread in the Middle East (eg United Arab Emirates, Saudi Arabia, Lebanon, and Israel), Africa (Libya, Morocco, South Africa and Egypt), and Asia (, China, Russia, Thailand and Taiwan).

El et al, (2011) detected 35% of OXA-48 carbapenem resistance harboring in *P. aeruginosa* in Belgium which showed different geographical distribution of OXA-48.

Katchanov et al, (2018) highlighted the burden of OXA-48 related with carbapenem resistance in Germany. They found 24.4% OXA-48 was responsible for carbapenem resistance which is higher as compare to current study which may be due to different geographical distribution.

Kazi et al, (2018) showed OXA-48 with 29.4% carbapenem resistance spreading in India. They found RT-PCR is highly accurate and rapid method to detect the carbapenem resistance. They also found the other class D beta lactamase enzymes like OXA-181 in India. This is little bit high rate as compare to our study which may be due to different area and trend of antibiotic use.

Borah, Saikia, and Hazarika (2016) reported OXA-48 β-lactamase gene detection in two organisms like *Escherichia coli* and *Pseudomonas aeruginosa* for very first time in Assam (India). They failed to detect β-lactamase phenotypically while they were successful in detecting OXA-48 by polymerase chain reaction. There are different genes, which exist genotypically but unable to detect phenotypically.

Ibrahim and Altayab (2017) showed 17.3% carbapenem resistant organisms harboring OXA-48 gene by PCR in Khartoum Afghanistan which agrees with current study.

Lee et al., (2015) detected OXA-48 carbapenemase in two organisms *E. coli* and *Klebsiella Pneumoniae*. This gene is also present in *Enterobacteriaceae,* in area of Afghanistan where presence of OXA-48 occurs more commonly. But alarming fact is that there is threat of spread of OXA-48 genes in other organisms through horizontal gene transfer in adjacent geographical area like Pakistan.

Begum and Shamsuzzaman (2016) showed the presence of OXA-48 in 20% cases in Dhaka Bangladesh. These results were almost similar to our study. They found this gene from microorganisms like *E coli, Klebsiella pneumonia* and C*itrobacter* in contrast to our study. This gene is also a genetic material of other lactose and non lactose fermenters like *P.aeruginosa.*

Braun et al., (2018) showed that different carbapenemase genes (like IMP-31, IMP-2, VIM-2, IMP-1, GES-2, IMP-13, IMP-15, IMP19, VIM-2, PER-1) were detected in *P. aeruginosa* by molecular micro-array based carbapenemase assay in Rawalpindi. In micro-array, the probes are short sequences designed to match parts of sequence of known or predicted open reading frames. This study exhibited 14% rate of carbapenemase genes which is near to our study rate of carbapenemase gene OXA-48 gene which was 16%. They isolated *P.aeruginosa* from pus, urine, throat swab, Foley catheter, blood, endotracheal tube, and bronchoalveolar lavage and nasogastric tube. In our study we isolate microorganism from pus samples only.

Mohamed et al., (2018) found 22.4% cases OXA-48 gene in strains of *P. aeruginosa* in Sudan quite near to our study. They explained that only one gene (OXA-48) is not responsible for mediating resistance towards antibiotics, other intrinsic and acquired genes are also involved.

Bakthavatchalam, Anandan and Veeraraghavan (2016) described OXA-48 carbapenemase out-break all over the World especially United Kingdom, Netherland, France, Germany and India.

Bouraf et al., (2017) first time detected the emergence of OXA-48 in Algeria. OXA-48 was reported in different organisms.

Dortet et al., (2015) found the dissemination of carbapenemases transcribing genes like OXA-48, KPC-2, NDM-1 and VIM-4 in Enterobacteriaceae in geographical region of Romania.

Mushi et al., (2014) identified carbapenemase transcribing genes in Tanzania. Different genes were detected in *Klebsiella pneumonia* (11%), in *P. aeruginosa* (10%) and *E coli* (8%) as in association of our study.

Scientific Novelty

The dramatic success of the pharmaceutical industry in the production of large number of antibiotics over the past four decades has caused contentment among the health care community about the treatment of infectious diseases. Despite all these antibiotics a

person could die in a hospital as a result of resistant bacterial infections. Bacteria have become resistant to antimicrobial agents as a result of chromosomal changes or the exchange of genetic material through plasmids and transposons which carry genes for antibiotic resistance. This crisis is fueled by extensive use of antibiotics in community as well as hospitals where there is injudicious use of antibiotics for treatment of common infections. Inter genus spread of resistance can occur in members of *Enterobacteriaceae*. A disaster is the appearance of *Pseudomonas aeruginosa* strains which are resistant to most of antibiotics and are significant cause of nosocomial infections. *Pseudomonas aeruginosa* is the foremost cause of serious infections particularly in those patients who have low level of white blood cells as a consequence of malignancy and chemotherapy. Resistance usually is a combination of poor penetration of antibiotics and the presence of enzymes that inactivate and alter the antibiotics. *Pseudomonas aeruginosa* is resistant to all groups of antibiotics β-lactams, Floroquinolones and Aminoglycosides. This resistance is continuing in alarming rate and the pharmaceutical industry and researchers cannot keep up producing novel antimicrobials to replace the existing ones against which the resistance is established and this has huge negative economic impact. According to reports the prevalence of beta lactamase producing strains are more than 80%. The last resort for treatment of beta lactamase producing is carbapenem. Carbapenems are commonly available antibiotics that have a wide range of antimicrobial activity and possess a exclusive structure that is described by a carbapenem coupled to a β-lactam ring which makes it resistant towards all kind of beta lactamases therefore it is the drug of choice against Gram negative rods like *P. aeruginosa.*

However the indiscriminate use of carbapenem to treat resistant cases has opted for carbapenemase generating organisms. In vivo inter genus transfer resistance between different organisms can occur and a patient can have the same and different types of beta lactamases. The genes for different beta lactamses have been reported in Pakistan like NDM, IMP, VIM and GES.

Despite the increasing rate of carbapenem resistance in Pakistan, there is still lack of a comprehensive understanding of the *P. aeruginosa* genes (OXA) that transcribe resistance.

Our study was the first to report the OXA-48 gene in strain of *P. aeruginosa* in hospitalized patients of PNS Shifa Karachi. Our results showed 16% carbapenem resistance as a result of OXA-48 gene which was detected by real time PCR.

According to our knowledge, this study is the first reporting OXA-48 incidence in Pakistan. Since there is no other study conducted and published in Pakistan, we assume that such strains carrying OXA-48 gene are present in other region of Pakistan. Based on other auther's observations, we also believe that OXA-48 gene could be transmitted from other *Pseudomonas aeruginosa*, injudicious antibiotic use and human travelling contributors to dissemination of these genes. Carbapenemase are prevalent in a particular geographical area but dissemination of resistant microorganisms can become faster and spread in large geographical regions and for that proper monitoring and surveillance should be implemented. We believe that our study will be valuable for detection of carbapenemase (OXA-48) in *P. aeruginosa* in the future.

CHAPTER 6

CONCLUSION

The prevalence of antibiotic resistance in infections caused by Gram negative rods in particular *P. aeruginosa* is alarmingly high. This high rate is due to the high production of ESBL (Extended spectrum beta lactamase) by these organisms. The last treatment options for ESBL is carbapenem, however the indiscriminate use of cabapenems have selected for more resistance in pathogens. We conclude antibiotic susceptibility test should be performed on every isolates of *Pseudomonas aeruginosa.*

Our results showed among three phenotypic methods like antibiotic susceptibility test, Modified Hodge Test and Modified carbapenem inactivation test, mCIM is the most superior one and we conclude that it can be recommended as Gold standard method in routine laboratories as a tool for carbapenemase detection.

Beta lactamases are transcribed by various genes like VIM, IMP, KPC, NDM and GES. These genes are transferred from resistant to susceptible strains and have been reported in various studies in Pakistan. Among these genes OXA-48 which transcribes class D was not previously reported or looked for *P. aeruginosa* isolates in Pakistan. Our study showed that OXA-48 gene is present in *Pseudomonas aeruginosa* isolates in our country. We conclude that bacteria can achieve substantial genetic diversity through acquisition and deletion and this effectively changes a non pathogenic population of bacteria into pathogenic one, which can disseminate at alarming rate, creating an antibiotic resistance crisis.

OXA-48 gene was detected by real time PCR using uniplex primers. It is concluded that in vivo transfer of resistance from one organism to other organism is occurring continuously and that the patient may be harboring the organisms that contain same or different genes transcribing carbapenemase as a result of gene exchange.

Prevalence rate of carbapenem resistance is 16% genotypically by real time PCR.

6.1 RECOMMENDATIONS

1. To reverse the alarming trend of multi-drug resistance in different pathogens which create microorganisms labeled as superbug, every person must work together in creating awareness regarding the indiscriminate use of antibiotics. On an individual level, physicians and surgeons and general public must take more responsibilities for the proper use of last resort drugs. On a global scale different countries need to make policies of antibiotic stewardship.
2. CDC proposes active screening for carbapenemase producing organisms. Failure in identification in carbapenem resistance leads towards improper antibiotic treatment and dissemination of pathogen strains. We recommend that there is a critical need for routine laboratories to inculcate methods for swift molecular detection of these genes that transcribe carbapenemase in Gram negative bacteria for control of infection, prevention and epidemiological surveys.
3. Existing methods of detecting resistant isolates in patients such as culture media followed by antibiotic susceptibility test are laborious time consuming and do not provide the data for presence of genes for epidemiologic surveillance. Therefore we recommend PCR based assay should be developed that identify carbapenemase genes either directly in patients samples or in cultures isolates. Unfortunately multiple carbapenemase genes are recognized and PCR based test normally can detect a small number of carbapenemase genes using multiplex primers.We therefore recommend that microarray based test should be developed that can at the same time identify the multitude of important carbapenemase genes as well as other important resistant genes for other antibiotics like aminoglycosides and fluoroquinolones.
4. Patients should also be educated to use the prescribed antibiotics in proper manner in order to increase patient compliance. These efforts will prevent the dissemination of pathogenic organisms on a large scale. Patients should follow the instructions of physicians. Failur to follow the instructions may not

kill the microorganisms. Misusing antibiotics by skipping doses or duration encourages the gradual emergence of resistant pathogens.

5. Public message should be conveyed by media to educate the people about the appropriate use of antibiotics. They should not use self prescribed antibiotics. Antibiotic should not be prescribed by common ailments.
6. Hand hygiene is a primary part of preventing multi-drug resistant organisms. Efforts should be made to promote staff ownership of hand hygiene.
7. People must stop the use of antibiotics for growth promotion and prophylactic ally disease prevention in animals.

6.2 STRENGTHS OF STUDY

1. To best of our knowledge this is the first study exploring the presence of OXA-48 associated with carbapenem resistance in *P. aeruginosa* strains in our country.
2. In our study antibiotic exposure was strength because we used only those isolates that were not exposed to any antibiotic, providing a more robust picture of antibiotic resistance towards carbapenem, study using isolates which have already exposed to antibiotics may miss a potential impact of use of different antibiotics and its association with resistance and data interpretation.
3. Our research explains a probable treatment protocol for *P. aeruginosa* infection. They provide substantial proof for restricting use of carbapenems in order to prevent the troublesome problems of resistance. The outcome will be, the patients will receive tailored antibiotic therapy reducing the risk of invasive infections and resistance transfer, grateful to the reduced selective antibiotic pressure.
4. In this study, we ssdetected carbapenem resistance and this will facilitate the physicians to modify therapy.
5. Timely and accurate detection of carbapenem resistance phenotypically can save patients lives.

6.3 LIMITATIONS OF STUDY

1. The foremost limitation of our study is that results cannot be generalized as we took data only from one Military set up where the subject population does not match the demograph of our country.
2. Our results may not even be applied at the same time to all bacterial isolates due to the dynamic bacterial genome with continuous activity of horizontal gene transfer occurs by conjugation. Yet this limitation shows the importance of continuous study of genotypic and phenotypic resistance. A microarray based PCR is used to detect genes and even its variants related with resistance directly from samples or culture but we could not utilize this technique as a result of limitations of resource.
3. There are multiple carbapenemase genes and their allelic variants we looked for only OXA-48 gene using uniplex primers. Ideally we should look for other carbapenemase resistant genes of *P. aeruginosa* strains at the same time also using multiplex PCR.
4. Only 16% of OXA-48 gene was detected because of small sample size.
5. This study included only hospitalized patients.

CHAPTER 7

7.1 REFERENCES

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(A)

FACULTY RESEARCH COMMITTEE
BAHRIA UNIVERSITY MEDICAL & DENTAL COLLEGE

LETTER OF APPROVAL

Ref No: FRC-BUMDC -01/ 2018-002

Date: 4th October 2018

To,
Dr. SHAISTA BAKHAT
M.Phil. Student
Department Of Pharmacology
BUMDC-Karachi

Subject: **APPROVAL OF SYNOPSIS**

The Faculty Research Committee has approved the synopsis of below mentioned student with modification in title of thesis as per suggestions of reviewer.

Student Name: **Dr. SHAISTA BAKHAT**

Title of Study: **"Prevalence of carbapenam resistant pseudomonas aeruginosa isolated by phenotypic and genotypic (OXA-48) methods in a tertiary care hospital".**

Further this letter is recommended and referred to ERC for approval on ethical grounds.

Regards

Assist Prof. Dr. Mehreen Lateef,
CO- CHAIRPERSON FRC-BUMDC

Cc:

FRC Record
PG Secretariat

BAHRIA UNIVERSITY MEDICAL AND DENTAL COLLEGE

Defence phase II, Sailor Street, adjacent to PNS Shifa, Karachi. Tel: 021-35319491-9

ETHICAL REVIEW COMMITTEE

LETTER OF APPROVAL

Dr. Shaista Bakhat
Lecturer
Department of Pathology
BUMDC-Karachi

Subject: Institutional Approval of research study

Title of Study: Prevalence of Carbapenem Resistant Pseudomonas Aeruginosa Isolated Phenotypic and Genotypic (OXA-48) Methods in tertiary Care Hospital.

Principal Investigator: Dr. Shaista Bakhat , Senior Lecturer Department of Pathology, Bahria University Medical and Dental College.

Reference No: ERC 43/2018

Dear Dr. Shaista Bakhat

Thank you for submitting the above mentioned study proposal. ERC Bahria University has reviewed this project in the meeting held on 24th-Sep-2018 and gives approval. Kindly notify us when the research is complete.

Regards,

PROF DR AMBREEN USMANI
Chairperson
BUMDC

Cc:
DG-BUMDC
Principal BUMDC
Chairperson ERC

ETHICAL REVIEW OF RESEARCH PROJECT

Proposed study "Prevalence of carbapenem resistant Pseudomonas aeruginosa isolated by phenotypic and genotypic (OXA-48) methods in tertiary care hospital." by Dr ① Shaista Bakhat ② PROF Dr Yasmin Taj ③ Surg Cdr Faisal Kaif has been reviewed and approved by Ethical Committee.

If any aspect of the work / project are changed, the matter is to be brought to the Ethical Committee for review and approval.

The grant of approval is only for the ethical aspects of the work. Issues of plagiarism, language and matters related to statistics and epidemiology have to be addressed by the relevant expects.

Dated: 28-9-18

SURG CDRE SHOAIB AHMED

Chair Ethical Review Committee

(Surg Cdre Shoaib Ahmed)

M KHURRAM AHMAD
Surg Cdre (Brig)
Asst Prof BUMS&DC
PNS SHIFA KARACHI

Member Ethical Review Committee

(Surg Cdre Muhammad Khurram Ahmed)

KASHIF RAZZAQ
Surg Lieut
Classified Medical Specialist
PNS SHIFA KARACHI

Member Ethical Review Committee

(Surg Cdr Kashif Razzaq PN)

SAMAN IJAZ
Surg Lieutenant PN
GDMO
PNS SHIFA, KARACHI

Member Ethical Review Committee

(Surg Lt Saman Ijaz PN)

(C) INFORMED CONSENT FORM

I am giving my consent to participate voluntarily and at my own will in this research project which aim to determine carbapanem resistant *Pseudomonas aeruginosa.*

I have been explained in detail the nature and significance of participating in the project and I understand the provided explanation.

I have been told that findings of my disease and my data will be kept strictly confidential and will be used only for the benefit of community, publications and paper presentations.

I have been explained that laboratory investigations will be conducted to check the resistance of antibiotic to certain organism. For this purpose I fully agree to give my sample (pus) at the beginning and end of study and when required in between.

I also agree to give all relevant information needed, in full and to the best of my knowledge to the researcher. It is clarified to me that no incentive will be provided to me for participating in the study, whereas I do have the right to withdraw from the study at any time.

I am advised to contact Dr. Shaista Bakhat on mobile number: 03322039311 or visit Shifa Hospital in case of any query/ emergency after having samples. They told me there will be no harm in providing blood, urine, sputum, and pus samples.

ID number_____Sex (Male, Female) _____

S/O, D/O, W/O, M/O, F/O_____

Signature / Thumb impression of Patient: _____

Name of Researcher: _____

Signature of Researcher: _____

Date: _____

مریض کے لئے اجازت نامہ

میں رضا کارانہ طور پر اس ریسرچ پراجیکٹ میں اپنی خوشی کے ساتھ حصہ لینے کا ارادہ کرتا ہوں۔اس پراجیکٹ کا مقصد کاربا پینم ریسسٹنٹ سوڈومیناس ایروگینوسا کا تعین کرنا ہے۔ مجھے اس پراجیکٹ کی اہمیت اور قسم کے بارے میں تفصیل سے آگاہی دی گئی ہے۔میں اس بات سے بھی آگاہ ہوں کہ میری مرض کے تشخیص اور اس کا ڈیٹا راز میں رکھا جائے گا اور یہ صرف کمیونٹی کے فائدے، اشاعیات اور پیپر پریذینٹیشن کے لئے استعمال ہوگا۔

مجھے اس بات سے بھی آگاہی دی جا چکی ہے کہ لیبارٹری کی انوسٹیگیشن کو بطور خاص نظام کے اینٹی بائیوٹیکس کی مزاحمت معلوم کرنے کے لئے استعمال کیا جائے گا۔اس مقصد کے لئے میں اپنے سیمپلز (خون، یورین، سپوٹیم، برانکیٹل لواج) کے لئے اس مطالعہ کا شروع، آخر اور جب کبھی ضرورت ہوئی دینے کے لئے رضامند ہوں۔میں اس بات سے بھی متفق ہوں کہ تمام معلومات پوری تفصیلات کے ساتھ ریسرچ کو فراہم کروں۔میں اس بات سے بھی بخوبی واقف ہوں کہ مجھے اس پراجیکٹ میں حصہ لینے کے لئے کوئی انعام نہیں دیا جائے گا۔جبکہ مجھے کسی بھی وقت اس مطالعہ سے ہٹنے کا اختیار ہے۔

مجھے اس بات کی نصیحت کی جاتی ہے کہ میں ڈاکٹر شائستہ بخت کو موبائل نمبر (0332-2039311) پر رابطہ کروں یا پھر پی این ایس شفا ہسپتال کے ایمرجنسی ڈیپارٹمنٹ کو (021-48506500) پر رابطہ کروں۔

انہوں نے مجھے بتایا ہے کہ خون، یورین، سپوٹم اور پس کے سیمپلز دینے میں کوئی مضائقہ نہیں۔

مریض کا نام_____جنس (مرد / عورت)، (بیٹا، بیٹی، بیوی، ماں، باپ وغیرہ)

دستخط_____

ریسرچ کے دستخط_____

تاریخ_____

(D)SUBJECT EVALUATION FORM

PRELIMINARY DATA
Date:_____ID No_____

Ward_____

Patient's name_____

W/O, S/O, D/O, F/O_____

Sex_____

Age_____Occupation_____

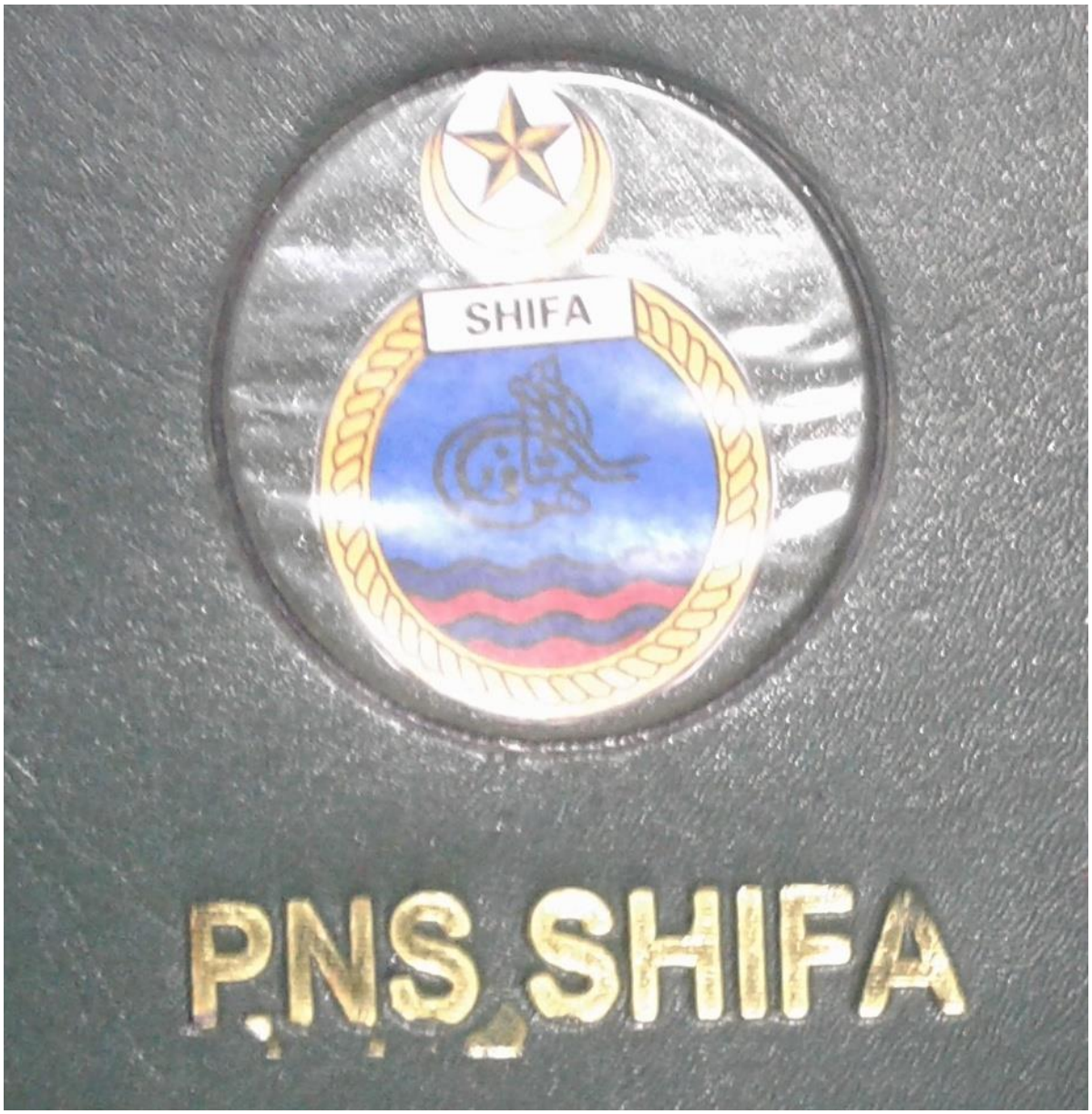
Address_____

Presenting complaints_____

Past History_____

Laboratory test:
Pus sample_____
Gram stainig_____
Bacterial Growth on blood agar_____
Bacterial Growth on MacConkey agar_____
Biochemical test (oxidase test)_____
Antibiotic sensitivity test_____
Modified Hodge Test_____
Modified Carbapenem inactivation test_____
Real Time PCR_____
ADVERSE EFFECTS
NA

(E) HOSPITAL CARD



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