Prevalence and Mechanism of Carbapenem-resistant Genes in Acinetobacter baumannii

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

Dedicated to my parents and siblings whose marvelous support and backing led me to this wonderful triumph

Declaration

I certify that this research work titled "**Prevalence and mechanism of carbapenem-resistant genes in Acinetobacter baumannii***"* is my work. The work has not been presented elsewhere for consideration. The objects that have been used from other sources it has been appropriately acknowledged/referred.

Signature of Student

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Abstract

Coccobacilli with a gram-negative Acinetobacter baumannii is a member of the ESKAPE group of free-living organisms, which are widely found in the environment in places like water, food, soil, sewage, animals, and humans. It is an opportunistic pathogen that has been implicated in numerous nosocomial infections across the globe. Carbapenem resistance has formed into a potential threat to humanity because of its rising prevalence among healthcare facilities. The present study was designed to characterize the presence of carbapenem-resistant genes including multidrug-resistant *Acinetobacter baumannii* strains, isolated from the tertiary care hospital in Pakistan. A biochemical test was performed for the initial identification of bacterial strains and further confirmed by MALDI-TOF mass spectrometry. Antibiotic susceptibility testing was done by using the standard Kirby-Bauer disk diffusion method, and carbapenem resistance determinants were further screened Metallo-beta-lactamase manufacture through Imipenem/Imipenem-EDTA-CDT. All 48 *A. baumannii* strains were further amplified by PCR for detection of OXA-51-like, OXA-23-like, and blaNDM genes. The OXA-51-like gene was detected in 46 strains (95.83%), while OXA-23-like was detected in 44 (91.66%) strains of carbapenem-resistant *Acinetobacter baumannii*. Moreover, New Dehli MBL, blaNDM gene was identified in four (8.33%) MBL positive carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates. All 4 positive blaNDM isolates were co-expressed by the OXA-23-like gene while OXA-51-like was absent in one blaNDM positive strain of carbapenem-resistant *Acinetobacter baumannii* isolates. A high prevalence of OXA-51-like, and OXA-23-like genes was seen among the carbapenem-resistant *Acinetobacter baumannii* isolates, followed by blaNDM1. The results of the study revealed that MDR Acinetobacter baumannii strains from two important cities in Pakistan (Rawalpindi and Islamabad) had a significant presence of carbapenem-resistant genes. This finding may result in clinical therapeutic failures.

Key Words: Acinetobacter baumannii, OXA, MBL, NDM, multidrug-resistant, AMR

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INTRODUCTION

Gram-negative, coccobacilli, Acinetobacter species belong to a nonparasitic, varied assembly of bacteria, and are frequently disseminated everywhere in location including, water, foods, soil, sewage, animals, and humans [1,2,3]. *A. baumannii,* an ESKAPE group of organisms, causes immune-compromised patients to experience higher rates of morbidity and mortality. [4,5,6].

Being a cunning pathogen, they are responsible for a wide range of ailments [3]. They typically appear on the skin [7], mucous membranes, lungs [8], digestive tract, and oropharynx [9,10,11]. Acinetobacter species, which select humid environments, were noticed on several human body parts, involving the nose, forehead, ear, trachea, throat, perineum, hand, and vagina. [12].

In addition, Acinetobacter species present in kind of diets and mammals, including poultry, fish, trout, fruits, milk, dairy products, and raw vegetables [13,15]. Furthermore, in a hospital environment including, ventilator tubing, plastic urinals, respirometers, humidifiers, sinks, mattresses, pillows, skin, and moist conditions in hospitals *Acinetobacter* species similarly recovered [11,15].

Since MDR Acinetobacter baumannii is typically a pathogen associated with hospital backgrounds, widespread studies have caught up it in epidemics and acquired infections such as sepsis, pneumonia, bacteremia, meningitis, and bladder infections. [14]. Additionally, outbursts often involve patients on mechanical ventilation and occur in ICUs and burn units [15].

The high prevalence of ICUs harboring this strain makes them responsible for infections that are linked to indwelling devices and that affect human organ systems, the respiratory and urinary tracts, and the peritoneal cavity. Due to their high mortality rate, they are a means of imposing acute illness on hospitalized patients. [3].

The intake of extended-spectrum antimicrobial agents becomes high by reason of severity of contaminations [5,15]. Thus, for their cure, imipenem, Sulbactam, Tigecycline, Tobramycin, Amikacin, and Colistin were the most recommended antibiotics. While intake of polymyxin, tetracyclines, glycylcyclines, quinolones, and aminoglycosides is not amazingly effective and

useful because of excessive dose and acquired resistivity against *A. baumannii*. However, this can lead to the classification of *A. baumannii* strains to multi, extensive, and pan-drug-resistant universal [5,16].

Acinetobacter species is considered as multidrug-resistant it shows resistivity to no less than four drugs classifications [3]. While according to an addition study, if Acinetobacter species is not susceptible to above two groups, including, ampicillin-sulbactam, fluoroquinolones, or aminoglycosides then it will declare as MDR [13,17,15]

However, if multidrug-resistant (MDR) Acinetobacter strains are additionally, resistant to carbapenems, then they will be considered as extensive drug-resistant (XDR). While pan drug resistant Acinetobacter spp. shows resistance to colistin and tetracycline. MDR A. Baumannii strain is known to be a major contributor to nosocomial infections. [18,15] and endangered the effectiveness of therapy of our current antibacterial agents.

In a previous study, it is thought to be a red alert pathogen [13]. Unfortunately, due to the high resistant profile of Acinetobacter, limited treatment options were available to effectively treat MDR Acinetobacter infections. However, a restricted range of antimicrobials worked for the therapy of nosocomial diseases. Due to the fact that it is becoming more common in healthcare settings, carbapenem resistance in MDR A. baumannii caused by class B, class C, and class D carbapenemase presents a risk to humanity [19,20].

Carbapenemase genes including, oxacillinase 51, oxacillinase 23, oxacillinase 24, oxacillinase 58, and oxacillinase 40 was stated a considerable process of drug resistance among MDR strains of Acenitobacter species [19]. However, blaNDM gene containing limited data is a newly found MBL in Pakistan.

LITERATURE REVIEW

2.1 Background of Acinetobacter species

In 1911, the genus Acinetobacter Micrococcus calcoaceticus was identified by Beijerinck from soil [21, 2] and has had different genus names for a very long time such as, mucosus, nitrates, Alcaligenes hemolysins, mucosus, Cytophaga, Neisseria winogradskyi Mimapolymorpha, M. calcoaceticus, Lingelsheimia, Bacterium anitratum, and Moraxella lwoffii, before becoming Acinetobacter [3,8].

2.2 Morphology and physical characteristics of Acinetobacter

Acinetobacter species are short and occurred in paired or extended, variable-length chains., typically 1.0–2.5 ml in size [3]. It is non-motile, and have typical shapes. Upon Leeds Acinetobacter medium, the colonies are pink, while Herellea agar has pale lavender colonies [2]. Being a non-lactose fermenter, Acinetobacter species has light lavender colonies on MacConkey agar.

Acinetobacter species have a GC content of 39% –47% [13] and are non-lactose-fermenter, Coccoco bacilli, indole negative, citrate positive, Gram-negative, and non-motile strains [1].

2.3 Natural habitation of Acinetobacter baumannii

A. baumannii can endure on soil and water, and transmit in hospital environments. Furthermore, medical equipments are means of transmission in intensive care units [15]. Mostly, these strains can remain feasible for more than 4 months longer than E.coli. Due to this ability, it can spread infections to hospitalized patients. Additionally, the soldiers acquire infections by Acinetobacter from hospitals, instead of the environment [15,22]. Acinetobacter spp. can frequently colonize, so it is difficult to control their spread from clinical materials as they are also present on non-living stuffs [15,23].

2.4 Virulence factor

Acinetobacter species have low virulence factors [1,15]. Surface hydrophobicity in them is bacterial adhesion, which protects it from phagocytosis [1,2,15]. Gram-negative Acinetobacter bacteria are pathogenic and cause antibiotic resistance due to several outer membrane proteins belonging to the OmpA family present in them [2,15]. A. baumannii endotoxins release pro-

inflammatory substances by stimulating the circulation of white blood cells thus, cause harm to neutrophils and lead to the inhibition of their migration, including their phagocytosis [1,2,15,24]. A. baumannii causes pathogenesis and destruction to host tissues, because of construction of exoenzymes [15,25].

2.5 Acinetobacter species threats

2.5.1 Pneumonia

Prevalence of Acenitobacter baumannii differs on one site to another. They are responsible for causing pneumonia as they are present in the respiratory tracts of hospitalized patients. Nosocomial pneumonia has a frequency of about 3%–5% in ICUs, while about 30%–75% crude death rate is being reported [2].

2.5.2 Bacteremia

Through intravascular and respiratory tract catheters, A. baumannii is also a means of causing bacteremia. It is less combated and rare from endocarditis through burns, and wounds [26]. Moreover, about 34.0% to 43.4% of death rates from A. baumannii due to bloodstream infections from ICUs [13,26].

2.5.3 Acute meningitis

Multidrug-resistant A. baumannii is involved in causing nosocomial post neurosurgical meningitis [2,27]. Several acute bacterial meningitis in adults were reported and it has a high mortality rate of about 70%, although its cause is often difficult to determine [13,27].

2.5.4 Additional exhibitions

Additionally, endocarditis was also seen as a host for A. baumannii in a few cases involving prosthetic valves. However, most infections including peritonitis, and keratitis were also cause by it [28,13]. Moreover, this strain also exists in water and soil [15] thus, causing human colonizers in the hospital [29]. Number of studies reported its in Europe, Hong Kong, Argentina, Brazil, China, North America, Japan, Taiwan, and Korea [15].

Moreover, pneumonia has been reported during the warm season [2,15]. However, in military personnel, an increased number of MDR Acinetobacter baumannii calcoaceticus complex was identified by UK and US [13].

2.5.5 Environmental factors

The environmental sites such as bedside tables, bed rails, pillows, mattresses, water, surrounding curtains, doors, cleaning equipment, computer keyboards are means of colonization and spread of A. baumannii in hospitalized patients [31]. Respiratory equipment contaminated by the hospital environment, used for mechanical ventilation leads to Acinetobacter outbreaks [31,3]. Moreover, according to another study contamination of feather pillows is a reason for the widespread of A. baumannii [32,15].

2.5.6 A. baumannii due to hand colonization

Acenitobacter baumannii in healthcare workers with damaged skin has high risks, as the spread of this strain to patients is also facilitated by the hands of staff [31]. However, excluding injured skin, about 3% and 23% of cases were reported by the epidemiological studies [26, 33].

2.6 Occupied patient in ICU

Acinetobacter can transmit to numerous patients nearby due to multiple-site patient colonization, broad environmental pollution, longevity on dry surfaces and hands, and antibiotic resistance [29]. Additionally, according to a study, out of 48 newly admitted patients to an ICU, 46 were colonized with A. baumannii [31]. However, A. baumannii spread is probable for colonized organisms in nosocomial infections due to an increased rate of MDR A. baumannii colonized patients in healthcare settings [34, 35,15].

2.7 Risk factors associated with A. baumannii

A. baumannii is medically an important Acinetobacter spp. responsible for increasing morbidity or mortality in critically ill patients [2]. Instead of community-acquired, these organisms are most often linked with nosocomial infections, as they are low virulence strains [36].

The naturally transformable group of Gram-negative bacteria known as Acinetobacter spp. is ideally adapted for genetic exchange. This crucial potential of Acinetobacter spp. to acquire primarily antibiotic resistant genes, seems plausible [37]. A hyper-transformable Acinetobacter strain was found in 1969, and it has been proven that DNA extracts from 265 bacterial strains belonging to eleven different genera may transform this particular strain. As members of the

genus Acinetobacter, these strains were also closely related genetically [38]. However, the main origins of CRAB are typically plasmids, transposons and integrons [39].

2.8 Antibiotic resistance mechanism

Class D, OXA carbapenemases among Acenitobacter strains were reported greatly in Scotland, Brazil, Spain, Cuba, Japan, France, Singapore, China, and Kuwait [45]. In A. baumannii, class B and class D enzymatic degradation is most prevailing mechanism of b-lactam resistance [36]. A. baumannii also has a prevalence of TEM-1 and TEM-2 but due to the strength of other resistance factors, their clinical importance is restricted [36].

Additionally, Metallo beta-lactams present on mobile genetic elements, cause a challenging threat due to their widespread among bacteria, [44]. However, Acinetobacter-derived cephalosporinases (ADCs) produced by A. baumannii, hydrolyze penicillin and cephalosporins, while, preserving the effectiveness of expanded spectrum cephalosporins when expressed at a normal range [45].

Furthermore, before introduction of resistance to imipenem, OXA23 was the first carbapenemase that was detected in 1985, in A. baumannii strains [45]. The enzymatic upregulation was initially determined by the presence of an insertion sequence ISAba1 and while about 28 beta-lactamase Acinetobacter-derived cephalosporinases genes were present in GenBank [40]. However, resistance to cefepime and carbapenems was acquired due to these enzymes in A. baumannii [45].

2.8.1 Antimicrobial resistance of A. baumannii

There are 5 major groups in which A. baumannii's antimicrobial resistance mechanisms fall [43]. Class 1 integrons commonly spread multidrug resistant genes in strains [41]. Nucleotidyltransferases, acetyltransferases, and phosphotransferase are previously described main classes of enzymes [3]. However, it was later found that an identified category of 16S rRNA methylases was involved in the mediation of 16S rRNA methylation, thus causing resistance to aminoglycosides [46]. Mostly the accountable genes are kept on transposons [46,3].

Additionally, 16S rRNA methylases from Korea, Japan, as well as the United States have been reported for Acenitobacter strains. While a fresh species of AME has only been detected and

associated with to amikacin resistance in Acinetobacter species in Japan, [31,3]. Alterations to the target ribosomal protein inside the cell are one type of resistance mechanism. [3].

A. Baumannii's quinolone resistance is mostly brought on by mutations in gyrA and parC genes, which alter DNA gyrase [42]. These modifications reduce the quinolones' affinity to bind to the enzyme-DNA complex. While efflux systems are a different method of quinolone resistance that lessens intracellular drug accumulation [40,47].

Polymyxins, which were initially identified in 1947, are the last resorts for pneumonia caused by MDR strains of Acenitobacter. Unfortunately, A. baumannii gained high treatment to colistin drug resistance comprising resistance mechanism [31]. While it is noted the that target site is responsible for reduced binding to Lipopolysaccharide for resistance case. Furthermore, less vulnerability to polymyxinse has been reported for Pseudomonas and changes in OMPs.

METHODS & MATERIALS

3.1 Study Design

The present study includes, bacteriological analysis, phenotypic, and genetic detection in Acinetobacter baumannii responsible for causing wounds, urinary tract infection, pneumonia, bloodstream infections in patients of different areas of Rawalpindi and Islamabad. Furthermore, AST and CDT were performed for further statistical analysis. This study was conducted at the bacteriology lab, NIH, Islamabad.

3.2 Sample collection:

In the present study, almost 80 samples including wounds, pus, urine, sputum, and blood were collected from February (2021) to May (2021) from different laboratories and hospitals in Rawalpindi and Islamabad. All samples were given numbers accordingly.

About 27 samples were collected from Citilab Rawalpindi, 17 from the NIH Islamabad, and 4 from AFIP, Rawalpindi. Moreover, samples are collected along with patient data including patient's gender, age, isolation sites, and IDs. All samples are shifted to bacteriology lab, National Institute of Health, Islamabad.

3.3 Culture Media Preparation and Autoclave:

The following culture media were prepared and used for detailed investigation of pathogenic Acinetobacter baumannii present in collected specimens, causing pneumonia, urinary tract infection, wounds, and bloodstream infection.

3.3.1 Brain Heart Infusion Agar (CM1032)

BHI agar facilitates micro-organisms growth due to its enriched medium property. Inoculation of samples was done on BHI agar initially. For its preparation, 7.4g of agar medium was added in 200ml of distilled water along with 3g of agar by heating on a Bunsen burners flame. The autoclave was done for sterilization.

3.3.2 MacConkey Agar (CM0115):

MacConkey Agar is a medium that facilitates the growth of all Enterobacteriaceae species. It was made by adding 26g of agar medium in 500ml of distilled water in a flask, and by heating it on a medium Bunsen burners flame. Moreover, 1.5g of agar agar was added to it to solidify and decontamination was done by autoclave.

3.3.3 Cystine lactose electrolyte deficient Medium (CM0301):

C.L.E.D is mostly used for growth of urinary pathogens. Its preparation was done by dissolving 10.8g of agar medium in 300ml of distilled water and by boiling it on a Bunsen burner flame. Autoclaving was done at the end.

3.3.4 Blood Agar

It is an enriched medium that contains about 5% of defibrinated mammalian blood and is used to grow fastidious organisms. It was made by suspending 28g of agar in 1litere of double distilled water and heating it to get fully dissolved. The dissolved agar was autoclave for 15 min.

3.3.5 Mueller Hinton Agar (CM0337):

Antimicrobial susceptibility test of Acinetobacter strains, detected in collected specimens was done by preparing Mueller Hinton agar. About 19g of MH agar was mixed in 500ml condensed water in a sterilized flask and 1.5g of agar-agar was also added to make it more solidify. After heating at medium flame, autoclaving was done at 121°C for 15 minutes.
15 minutes autoclave at 121 C was done under 15 pressure for sterilization of all media. After autoclave is turned off and then allow to cool down for 30 minutes.

3.3.6 EDTA solution preparation:

EDTA solution is used in combination with an imipenem disk for the detection Metallo-beta-lactamase. The solution was prepared by mixing 18.61g of EDTA for 100ml of double distilled water in a flask, and then NaOH was also added and pH was adjusted at 8. Furthermore, overnight stirring was done on a magnetic stirrer, and the solution was autoclaved for 15 min at 121C.

3.4 Pouring and Incubation:

The sterilized media was cooled down for about 20 minutes. Before pouring, the benchtop was cleaned with 70% alcohol. About 25ml of the sterilized medium was poured into the petri-dish by keeping the flame on to avoid contamination. After pouring, the media was left for about 20 minutes to cool down and solidify.

Furthermore, incubation was done by keeping the Petri-dishes with the medium in an incubator 24 hr. incubation was done to confirm that the prepared medium was pure and had no growth due to contamination during pouring.

3.5 Streaking and Incubation:

Streaking was done to isolate pure colonies of pathogenic bacteria from collected specimens. All the specimens were inoculated on appropriate culture media. The blood specimen was streaked on blood, MacConkey, and CLED media after 24 hours of incubation in brain heart broth (BHI) at 37°C. Pus specimen was inoculated on MacConkey's agar and CLED agar, and sputum was inoculated on Blood and CLED agar. Streaking was done by using a sterilized wire loop in a single and continuous streaking manner. All the process was carried out in a biosafety cabinet to avoid contamination. In addition, the streaked plates were incubated for 1 day to get colonies

3.6 Identification and Characterization of Acinetobacter baumannii

After overnight incubation, morphological and biochemical characterization of bacterial strains were performed. The presence of Acinetobacter baumannii was confirmed by several tests including oxidase, Indole, methyl red, catalase, VP, gram staining, morphology, smell, MALDI Mass Spectrometry Time of Flight.

3.6.1 Physical Identification

After 24 hours of incubation, the bacterial colonies were identified by their physical appearance including their color, smell, and morphology.

3.6.1.1 Microscopy:

In microscopy, Gram staining was done to examine bacterial form, color, shape, and structure by preparing a film or a smear. Smear is made by mixing fresh bacterial colony in saline with the help of a sterilized wire loop. Then the prepared smear was fixed by heating on a Bunsen burner and left to be dried.

Staining was done by using crystal violet, safranine, decolorizer, and iodine crystal. Each stain was left for 1 minute except decolorizer i.e., 30 secs. Every stain was washed with tap water and the dried slide was seen under a microscope with a 100-oil emulsion lens.

3.6.1.2 Catalase Test

A catalase test was performed to identify bacteria that produce catalase enzymes. It was done with a 3% H_2O_2 solution on a glass slide by applying a bacterial colony on it with the help of a sterilized wire loop. Bubbles were produced in this test.

3.6.1.3 Oxidase Test

This test was done to determine bacterial colonies that produce cytochrome c oxidase enzyme by putting purple color oxidase reagent (4N Tetra methyl P phenyl diamine HCL) on Whatman filter paper along with bacterial colony.
Furthermore, a bacterial strain from the cultured plate was smeared on filter paper using a sterilized wire loop. The result was taken within 10 secs.

3.6.1.4 Indole Test

This test identifies gram-negative bacteria on the bases of indole production due to the breakdown of tryptophan amino acids by a few tryptophanase enzymes. This test includes peptone water as a nutrient medium in an indole test tube in which a bacterial colony was inoculated with a sterilized wire loop. After 24 hours incubation, 1 drop of indole reagent was added and results were taken after 2 to 3 minutes.

3.6.1.5 Methyl Red Test

MR test was performed to determine the low glucose fermentation level in Acinetobacter baumannii. Low glucose fermentation was indicated by yellow color while isolates with high glucose fermentation levels produced red.

MR test was performed by inoculating a fresh colony of Acinetobacter baumannii in peptone buffer with a sterile wire loop. The inoculated tube was incubated overnight at 37°C. Following incubation, 4-5 drops of pH indicator methyl red were added to the cultured tube. Results were recorded as positive (red) and negative (yellow).

3.6.1.6 Voges-Proskauer Test

The production of acetyl methyl carbinol was examined by this test. Isolates with low glucose fermentation were indicated as negative.

For the VP test pure, a fresh colony of the isolate was inoculated in the broth by using a sterile wire loop. Then overnight incubation was done aerobically. Following incubation, six drops of solution A containing alpha-naphthol, and two drops of solution B containing 40% potassium hydroxide were mixed thoroughly in a tube. Results were recorded as positive (pink color ring at the surface) and negative (colorless/yellow ring).

3.7 MALDI time of flight mass spectrometry

3.7.1 Principle

It is precise, fast, and cost-effective method for confirmation of microorganisms. The co-crystallized bacterial sample within the matrix is ionized by a laser beam and results in desorption and ionization, due to which protonated ions were generated. Then these protonated ions were separated on the bases of their mass and electric charge in the vacuum and analyzed by a time-of-flight analyzer. The time-of-flight data generates peptide mass fingerprint for charged analytes of the unknown organism, which can be compared with mass spectra of known microbial isolates contained in the database (Escherichia coli ATCC8739 control strain) and rapidly identifies the organism.

3.7.2 Requirements

Gloves, Blood agar, CLED agar, and MacConkey agar, bacterial colony, MALDI slide, α-cyano-4-hydroxycinnamic acid matrix, E.coli ATCC8739 control strain, and sterile inoculating 1ul loop were required for MALDI-TOF-MS test.

3.7.3 Sample preparation process

The sample was prepared by direct cell profiling method, in which the biological specimen sub-cultured on blood agar, MacConkey agar, and CLED agar. A single colony of bacteria from a sub-cultured microbial sample was picked up through a sterile inoculating disposable 1ul loop and spotted directly on the sample slide. Furthermore, 1ul of the matrix was spread on the sample and leftover for air drying. The sample slide was loaded in the Vitex and the result was recorded in software (MYLA software) within 2 hours.

3.8 Antimicrobial-Susceptibility Test

An antimicrobial susceptibility test is done by the Kirby Bauer disk diffusion method to identify MDR strains of Acinetobacter. A pure and single colony of fresh Acinetobacter baumannii was transferred to sterile normal saline by using a sterile wire loop. A fixed turbidity of 0.5 McFarland standard was used. The sterile cotton swab was dipped into the bacterial suspension and rotated multiple times after 15 minutes of inoculum suspension adjustment. The sterile cotton swab was then used to apply hard pressure to the interior walls of the suspension tube above the liquid level to remove any excess liquid. Furthermore, Muller-Hinton agar plates were dried for about 1 hour in the incubator at 60°C before streaking. Then inoculation was done on the dried surface the of MH agar plate. The process was repeated three times by turning MH agar plate at 60°C to confirm uniform spread of the inoculum. Once dried, antibiotic disc were inserted on the surface and pressed gently with sterile forceps to confirm that disks were attached to the agar surface.

3.8.1 Kirby-Bauer disk diffusion susceptibility test

By standardized Kirby-Bauer disk diffusion test, the susceptibility of drugs against Acinetobacter baumannii was determined. The sensitivity and resistance of selective antibiotics against the Acinetobacter baumannii were determined by measuring different zones of inhibition in millimeters according to the CLSI principle. The suspension of Acinetobacter baumannii was inoculated evenly on MH agar plate and left for drying. Then the following antimicrobials were applied against Acinetobacter baumannii on the MHA plate, cefepime (FEP), trimethoprim/sulfamethoxazole (SXT), amikacin (AK), ticarcillin (TIC), colistin (CS/CT), meropenem (MRP), imipenem (IMP), ciprofloxacin, Ertapenem (ETP), gentamicin (CN), ampicillin (AMP), Tigecycline (TGC), doxycycline (DO), and Tetracycline. Moreover, the plate was incubated for about 18 hours. Then inhibition zones were measured in millimeters

via referring to the ranges suggested in FDA and CLSI. The susceptibility results were interpreted as resistant, sensitive, or intermediated (Table 1).

Table 1: Zones of inhibition against different antimicrobial disks

Antimicrobial disks	**Disk content**	**Zone diameter breakpoints Sensitive**	**intermediate**	**Resistant**
Cefepime	30µg	≥18	15-17	≤14
Trimethoprim/sulfamethoxazole	25µg	≥16	11-15	≤10
Amikacin	30µg	≥ 17	15-16	≤ 14
Ticarcillin	75µg	≥20	15-19	≤14
Meropenem	10µg	≥ 16	14-15	≤ 13
Imipenem	10µg	≥16	14-15	≤ 13
Ampicillin	10µg	≥17	14-16	≤13
Gentamicin	10µg	≥15	13-14	≤12
Ciprofloxacin	5µg	≥ 21	16-20	≤ 15
Tetracycline	30µg	≥15	12-14	≤11
Doxycycline	30 µg	≥13	10-12	≤9
Tigecycline	15 µg	≥16	13-15	≤12

3.8.2 Combined-Disk Test

The isolates of gram-negative Acinetobacter baumannii showing resistance to meropenem and imipenem were screened through CDT. It is an inexpensive and significant technique commonly used in the laboratory for the phenotypic screening of MBL. The combined disk of imipenem (10µg) and Ethylenediaminetetraacetic acid were prepared as described previously [13].

0.5M Ethylenediaminetetraacetic acid disodium salt (EDTA) stock solution was made by dissolving 186.1 g of disodium EDTA in 100 ml of double distilled water in a beaker.
Then to increase the solubility of EDTA, the pH of double distilled water was increased to 8.0 by adding approximately 20g of NaOH. After measuring the pH with a pH meter, the beaker was placed on the magnetic stirrer along with a magnetic pellet and stirred for about 18 to 24 hours. Once the EDTA was fully dissolved, the solution was transferred into the cylinder and sterilized by autoclaving.

3.8.2.1 Imipenem /Imipenem-EDTA Combined Disk Test

A fresh colony of carbapenem-resistant Acinetobacter baumannii was mixed into the sterile normal saline with the help of a sterilized wire loop. Then after fixing the turbidity (106 CFU/mL), the inoculum was inoculated over the MH media plate. After swabbing, the MH agar plate was left to dry. Then, two imipenem disks of 10µg each, were placed firmly on MH plate by using sterile forceps at 20mm. Moreover, using a micropipette, 5l of prepared stock solution of EDTA was added to one of these two imipenem discs to achieve the necessary concentration of 750g. Then the MH agar plate containing Imipenem-Imipenem/EDTA disks was incubated overnight and results were recorded.

3.9 Extraction of bacterial DNA

DNA template of Acinetobacter was extracted at room temperature with the help of a micropipette. Extraction was done by using QIAamp DNA mini kit. Then, a single, fresh strain of multidrug resistant Acinetobacter was taken with the help of a sterile loop and mixed well in the ATL buffer. ATL buffer was used to lyse tissue and stored at 15-25°C. After spinning, 20 microliter proteinase k was mixed, and the solution was placed in the water bath overnight at 70°C. Then the Eppendorf tube containing the solution was vortex again before and after adding the AL lysing buffer to it. Following a 10-minute incubation period at 70°C, the solution was vortexed once again. Then, centrifugation was done for about 1 minute (8000rpm) before

and after adding ethanol in mixture. The tube was replaced with a new collection tube (2ml) by discarding the filtrate and 500 microliter of washing buffer AW1 was added to it. Then again, after centrifugation for 1 min at 8000 rpm, 500 microliter of washing buffer AW2 was added. Repeatedly, after centrifugation, QIAamp mini column was replaced with an Eppendorf and 100 microliters of elusion AE was added to it. In end, centrifugation was done for 3 minutes at 10,000 rpm and purity of DNA was checked through nanodrop spectrophotometer and, then DNA was kept at -20C.

3.10 Primers

Primers were selected for oxa51-like NDM, and oxa23-like genes (Table 2). Primer sequences were finalized by testing on NCBI blast. The forward and reverse primer (5'-3') sequences of all genes were also analyzed by oligo-calculator and oligo-evaluator (Table 3). Primers were selected based on their GC content (40-60%), melting temperature (45-60°C), and nucleotides length (18-25).

Table 2: Primers for oxa-51-like, blaNDM, and oxa-23-like genes in carbapenem-resistant Acinetobacter baumannii

Primer	**Sequence (5′–3′)**	**Gene Product**	**size (bp)**	**Reference**
NMD-F **NDM-R**	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	blaNDM	621	[48]
OXA-51-like F **OXA-51-like R**	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	OXA-51-like	353	[49]
OXA-23-like F **OXA-23-like R**	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	OXA-23-like	501	[49]

Table 3: Length, melting temperature, and GC content of blaNDM, and oxacillinase genes after analysis on oligo-calc

Sequence type	**Oligo Type**	**Base Count**	**Length (bp)**	**Tm (°C)**	**GC%**
DNA	NDM-F	A = 1, U = 0, G = 7, C = 3, T = 9, I = 0	20	56	50.0
DNA	NDM-R	A = 5, U = 0, G = 5, C = 6, T = 4, I = 0	20	58	55.0
DNA	OXA-51-like F	A = 3, U = 0, G = 5, C = 4, T = 8, I = 0	20	54	45.0
DNA	OXA-51-like R	A = 3, U = 0, G = 5, C = 4, T = 8, I = 0	20	54	45.0
DNA	OXA-23-like F	A = 7, U = 0, G = 7, C = 3, T = 3, I = 0	20	56	50.0
DNA	OXA-23-like R	A = 7, U = 0, G = 7, C = 3, T = 3, I = 0	20	52	40.0

3.11 Polymerase-Chain-Reaction Assay

The 25 microliter PCR reaction was made by combining forward and reverse primer templates (1 ul each), 5 microliter of extracted DNA, and 13 microliter of frozen HotStarTaq DNA Polymerase Master Mix in a 0.2 ml thin-walled PCR tube. Each gene's primer template was generated independently for the reaction.

3.11.1 PCR Amplification

The reaction containing PCR tubes was placed in conventional PCR for thermal cycling. The PCR was optimized for blaNDM gene reaction mixture at 57◦C (621bp), OXA-51-like gene reaction mixture at 57◦C (353bp), and OXA-23-like gene reaction mixture at 58◦C (501bp). The PCR process was initiated by a denaturation step and ends at extention (Table 4).

Table 4: Thermal cycling conditions for amplification of carbapenem-resistant genes

Carbapenemase class	**Gene**	**Thermal cycling conditions**				
		Initial denaturation	**30 cycles**			**Final extension**
			denaturation	**Annealing**	**Extension**	
Class D carbapenemases	OXA-51-like	95◦C/10min	95◦C/1min	57◦C/1min	72◦C/1min	72◦C/5min
	OXA-23-like	95◦C/10min	95◦C/1min	58◦C/1min	72◦C/1min	72◦C/5min
Class B carbapenemase	blaNDM	95◦C/10min	95◦C/1min	57◦C/1min	72◦C/1min	72◦C/5min

3.12 Gel Electrophoresis procedure

The gel was prepared by adding agarose powder to distilled water and mixed by heating in the microwave. The solution was cooled down at 55°C for 5 min and shifted into a tray. The electrophoresis comb was inserted in the agarose buffer to form wells after solidification. Furthermore, the solidified gel was submerged into an anode and cathode containing an electrophoresis chamber that was 1/3 filled with TBE buffer. TBE buffer maintains pH and conducts electricity.

3.12.1 Sample Preparation for gel electrophoresis

The sample was prepared for electrophoresis by mixing 2 µl of DNA loading dye in a 5 µl PCR product. 5 µl of ethidium bromide was added. Then the sample was loaded in the wells along with a ladder (Thermo Scientific GeneRuler100bp) by using a sterile micropipette. The power supply was connected to the 2 chambers that were set at 102m A, 10 Watts, and 100volts to run the gel. The process ended after 40 min.

3.13 Image Visualization

Bands of blaNDM, oxa51 and oxa23 genes were visualized under high resolution imaging system and their base pair size i.e 621, 353, 501 was determined by comparing to DNA ladder (100bp).

RESULTS

Among 80 collected samples, 48 samples from different sources had Acinetobacter baumannii in them. Among them,17 samples were from the NIH Islamabad, 27 from CITI Laboratory, and 4 from AFIP Rawalpindi. Rawalpindi. Selected specimens were based on different origin, including pus (n= 6), urine (n= 5), blood (n= 26), tracheal secretion (n= 1), and sputum (n= 10) specimens. Patients' ages ranged from 17 days to 75 years.

After overnight incubation at 37C, Opaque and smooth colonies grew on nutrient agar were lactose fermenter gram-negative Acinetobacter as shown in Fig.1.

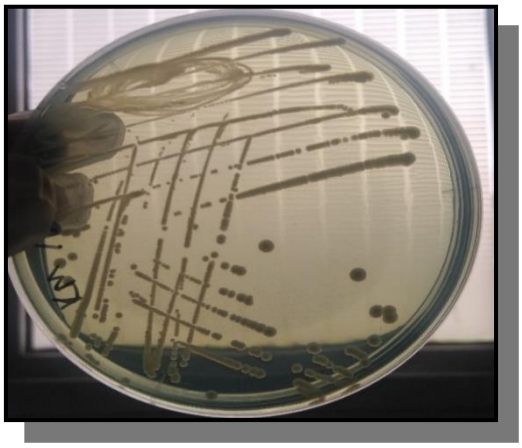

Figure1: Colonies of gram-negative Acenitobacter strains grown on CLED agar plate after overnight incubation

A catalase test was done to identify catalase-producing A.baumannii. Breakdown of hydrogen peroxide into hydrogen and oxygen results in bubble formation. All 46 isolates of A.baumannii were catalase positive. (Figure 2).

In gram-staining, single and paired, pink-colored colonies of gram-negative coccobacilli were revealed as Acinetobacter baumannii (Figure 3).

During oxidase test, instead of the dark purple color indophenol, the yellow color was seen within 10 sec. All 46 isolates were tested negative for the oxidase test (Figure 5).

Indole test was conducted to check indole production due to existence of tryptophanase enzyme. All isolates of A.baumannii were tested negative (Figure 4).

Glucose fermentation level was determined by a methyl red test. All 46 isolates were tested negative due to a low level of Glucose fermentation. The yellow color was produced in all overnight incubated tubes of isolates (Figure 7).

All 46 isolates of A.baumannii were identified by the Voges-Proskauer test (Figure 7). Instead of a red, a yellow ring was formed at the surface for all isolates inoculated in tubes. 100% negative result was recorded. (Table 5).

Table 5 The biochemical characteristics of Acinetobacter baumannii strains

Biochemical Tests	**Acinetobacter baumannii (n=48) (%)**	
Morphology	Coccobacilli	100%
Catalase	+	100%
Oxidase	_	100%
Indole	_	100%
Methyl Red	_	100%
Voges-Proskauer	_	100%

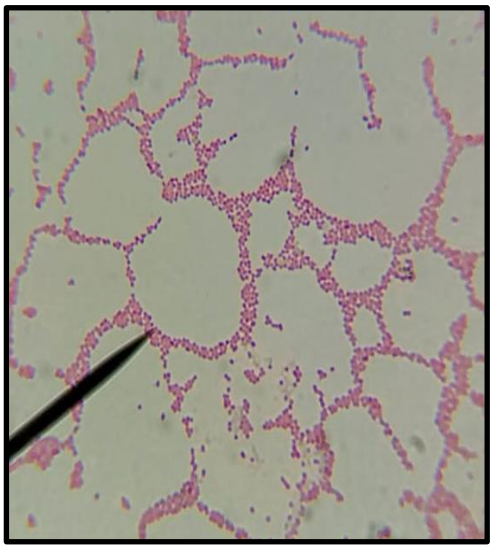

Figure 3: Acinetobacter baumannii-gram-negative coccobacilli arranged singly or in pairs

Figure 2: Positive Catalase test showing the emission of oxygen gas.

Figure 5: A negative oxidase test (yellow colored) for all A.baumannii isolates.

Figure 4: Negative indole test showing the presence of A.baumannii

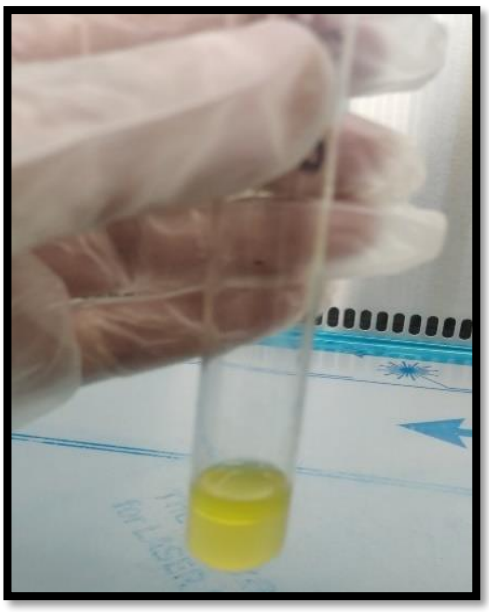

Figure 6: Voges-Proskauer test tested negative

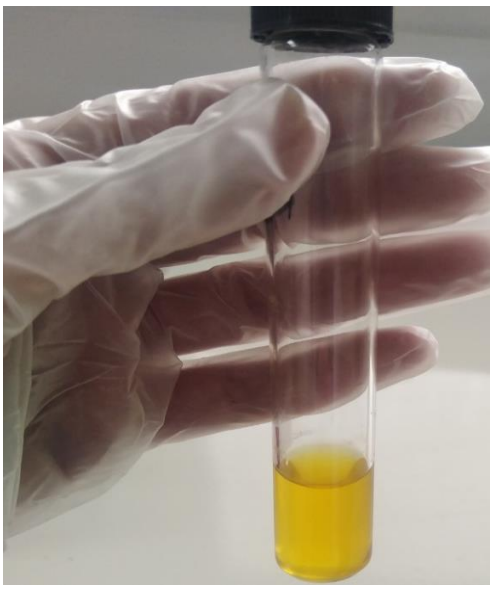

Figure 7: Negative methyl red test showing low Glucose fermentation level

All samples were confirmed by the VITEK (MALDI TOF MS) technique, by using fresh colonies along with control strain (E.coli ATCC8739). Results were recorded after 2 hours as shown in (Figure 8).

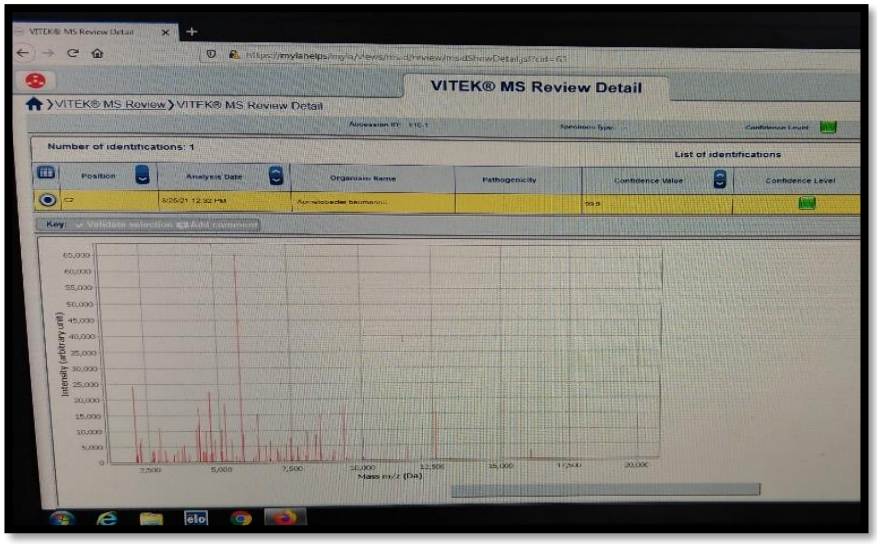

Figure 8: 99.9% confirmed identification of Acinetobacter baumannii by using VITEK MALDI TOF MS. x-axis represents the mass to charge ratio of 2500-20,000 and the y-axis presents intensity in arbitrary units ranging from 0-65,000.

AST of total twelve disks was performed for A.baumannii (Figure 9). The zone diameter for cefepime (30µg), ampicillin, amikacin (30µg), ticarcillin (75µg), CIP, meropenem (10µg), gentamicin (10µg), TG, TE, doxycycline (30µg), IMP, and trimethoprim/sulfamethoxazole (25µg) were recorded by CLSI principle (Figure 10).

Figure 9: Antimicrobial disks used for AST

Figure 10: Different antibiotics showing different sensitivity/resistance against A.baumannii.

In the present study, 48 multidrug resistant strains of Acenitobacter were collected clinically (Table 3). Out of these strains, 95.83% showed resistance to imipenem, and were also resistant to cefepime, amikacin, meropenem, and ampicillin.

All imipenem resistant strains showed resistance to sulfamethoxazole/trimethoprim (87.50%), ticarcillin (85.42%), gentamicin (72.92%), ciprofloxacin (83.33%), tetracycline (83.33%), doxycycline (56.25%), and tigecycline (58.33%), respectively.

Moreover, in current study, the susceptibility pattern was 4.16 percent for imipenem, 12.50 percent for sulfamethoxazole/trimethoprim, 14.50 percent for ticarcillin, 16.66 percent for gentamicin, 4.15 percent for meropenem, and 6.25 percent for ciprofloxacin. (Figure 11).

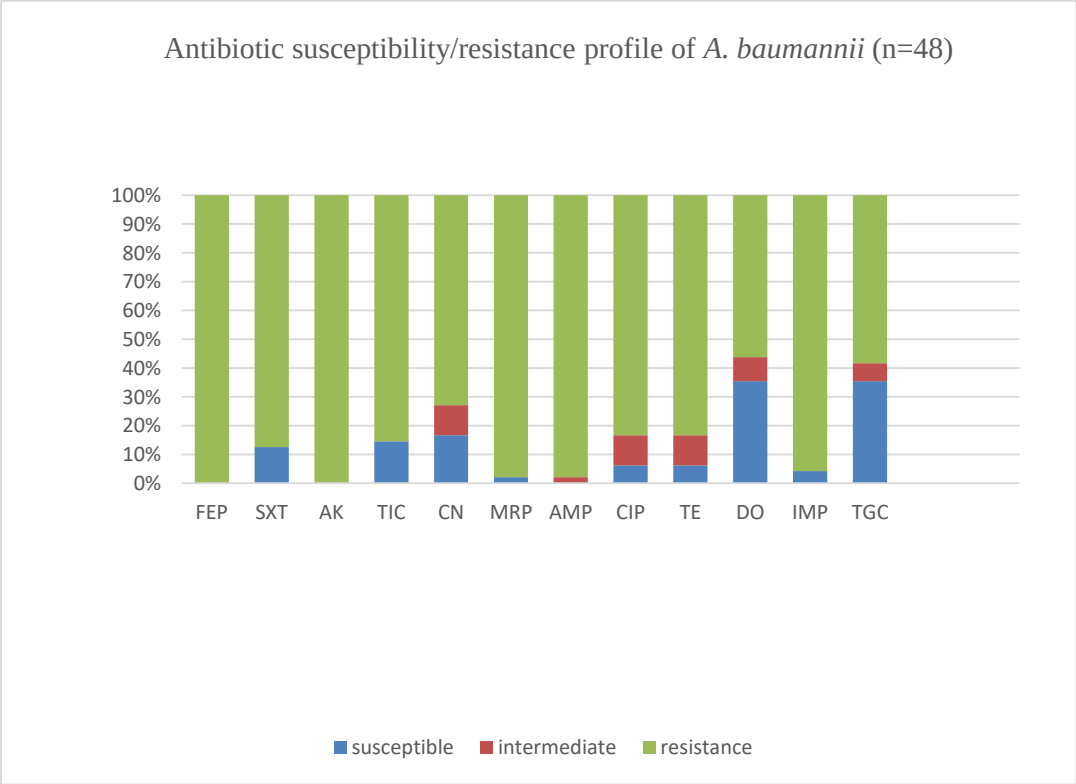

Figure 11: Antimicrobial Resistant, Intermediate and Susceptibility pattern
FEP= cefepime, SXT= trimethoprim-sulphamethoxazole, AK= amikacin, TIC= ticarcillin, CN= gentamicin, MRP= meropenem, AMP= ampicillin, CIP= ciprofloxacin, TE= tetracycline, DO= doxycycline, IMP= imipenem, TGC= tigecycline

Table 6: Antibiotic Susceptibility Pattern of Carbapenem-resistant A. baumannii isolates.

Antimicrobial agents	Susceptible (%)	Intermediate (%)	Resistance (%)
FEP	0	0	100
SXT	12.50	0	87.50
AK	0	0	100
TIC	14.50	0	85.42
CN	16.66	10.42	72.92
MRP	2.08	0	97.91
AMP	0	2.08	97.91
CIP	6.25	10.42	83.33
TE	6.25	10.42	83.33
DO	35.41	8.33	56.25
IMP	4.16	0	95.83
TGC	35.41	6.25	58.33

A CDT was performed to verify the production of the MBL enzyme (Figure 12). Four strains were MBL negative while 91.64% shows positive results as shown in Figure 13.

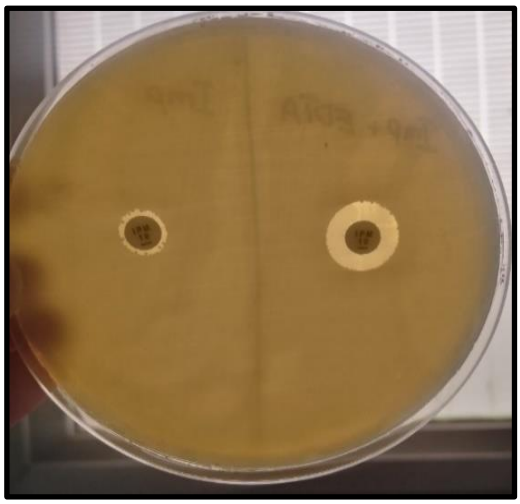

Figure 13: IMP-IMP/EDTA combined disk test

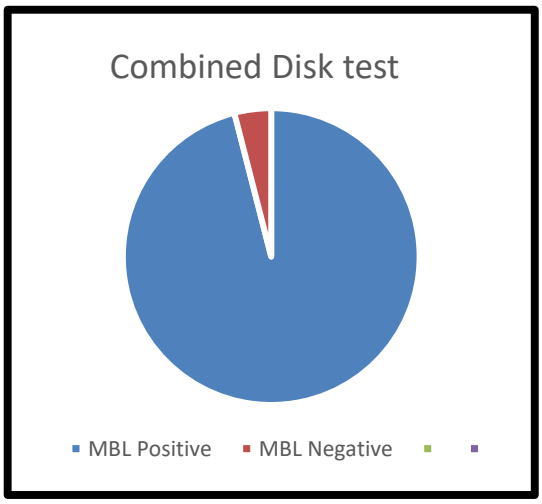

Figure 12: CDT for IMP resistant MBL positive and MBL negative strains

The carbapenemase genes including oxacillinase 51 and oxacillinase 23 and NDM were spotted by using PCR (Figure 14). PCR results showed that 93.75% carried the oxacillinase 51 genes, while the oxacillinase 23 gene was present in 91.66% of strains. However, 9.99 percent of isolates, blaNDM gene was identified (Figure 15). PCR results showed that out of 46 isolates that were phenotypically carbapenem-resistant 44 isolates contained genes for both oxa51 and oxa23. While out of 2 isolates showing phenotypically sensitivity to carbapenem, only 1 carries the OXA-51-like gene. The oxa23 and blaNDM showed resistance to carbapenem, amikacin, tetracycline, cefepime, and trimethoprim-sulfamethoxazole was shown by all oxa23 and NDM genes. However, all were susceptible to tigecycline. Furthermore, carbapenem-resistant strains showing expression of both OXA-23-like and blaNDM genes have been sent to Finland for whole-genome sequences for further analysis.

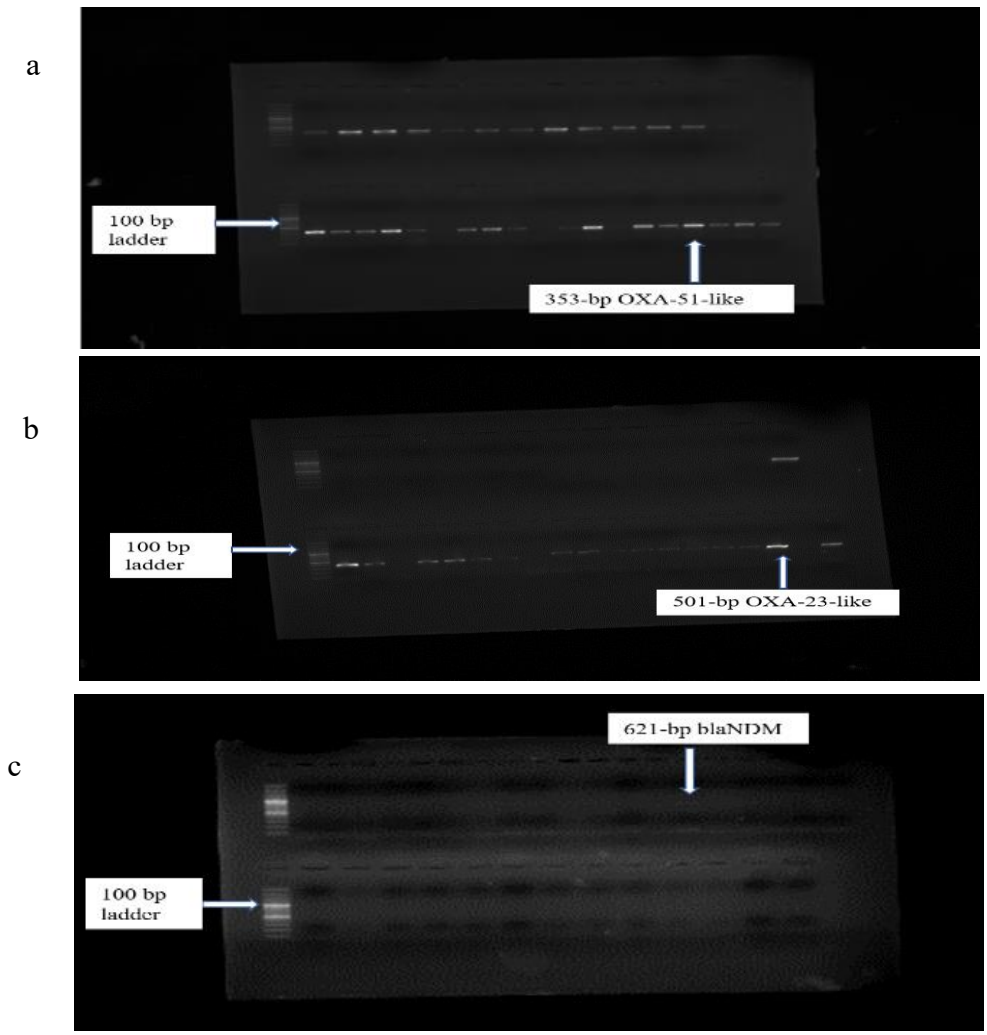

Figure 14: Valuation of PCR assay for rapid recognition of carbapenem-resistant genes for *A. baumannii* isolates a. Result of multiplex PCR detecting OXA-51-like gene, positive bands were observed on 353bp ladder b. The result of multiplex PCR detecting OXA-23-like, positive bands of OXA-23-like gene was observed on 501bp ladder c. Result of multiplex PCR detecting the blaNDM gene, positive bands of the blaNDM gene was observed on the 621bp ladder. The ladder used is the Gene ruler 100bp (Thermo Scientific)

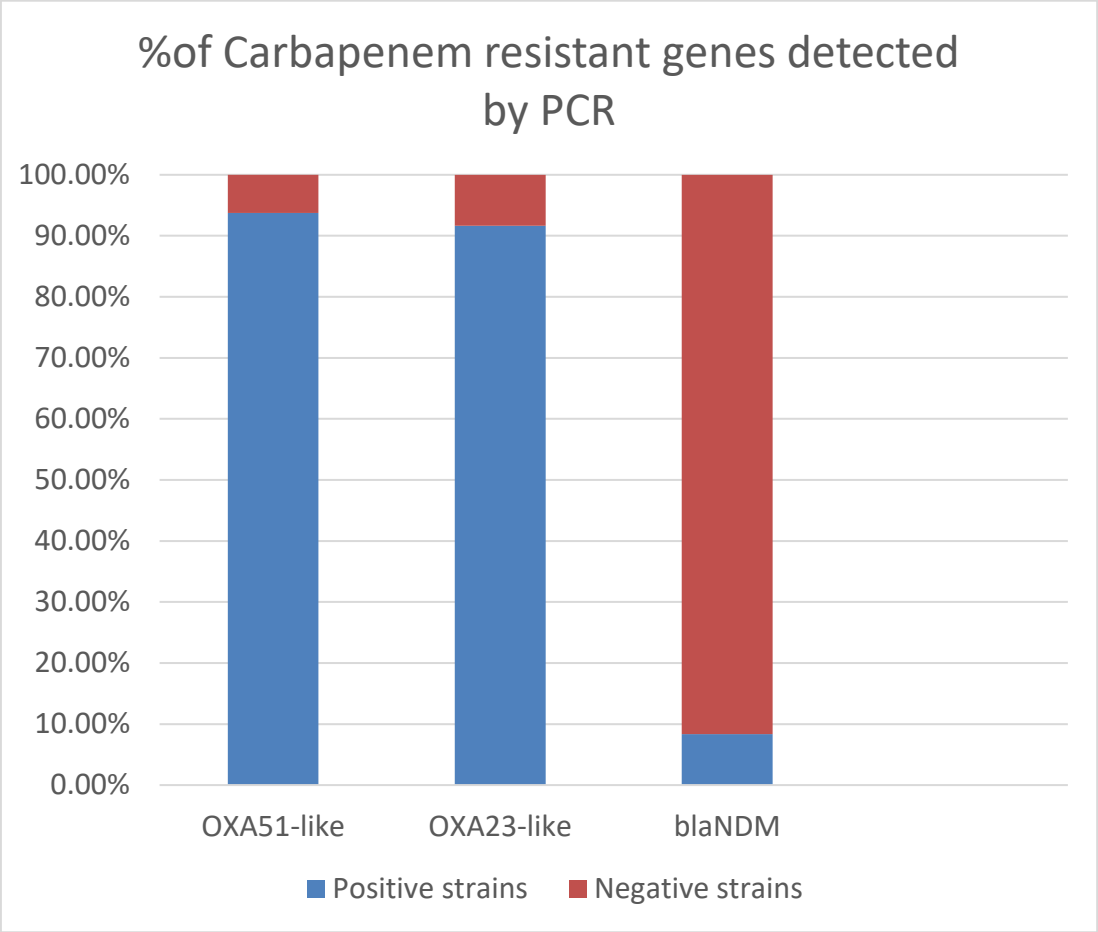

Figure 15: % of OXA51-like, OXA23-like, and blaNDM genes in Carbapenem-resistant strains.

Table 7: Sample ID's and prevalence of genes

Sample ID's	blaNDM	OXA-51	OXA-23
K11	_	POS	POS
K2	_	POS	POS
N10	POS	_	POS
K10	_	_	POS
K7	_	POS	POS
2B	_	POS	POS
K6	_	POS	POS
29	_	POS	POS
N7	_	POS	_
N9	_	_	_
18A	_	POS	POS
29A	_	POS	POS
25A	_	POS	POS
4A	_	POS	POS
27	_	POS	_
K01	_	POS	POS
	_	POS	POS
K02	_	POS	POS
K03	_	POS	POS
K04	_	POS	POS
K05	_	POS	_
K09	POS	POS	POS
k8	POS	POS	POS
52	_	POS	POS
53	POS	POS	POS
55	_	POS	POS
77	_	POS	POS
5	_	POS	POS

Sample ID's	blaNDM	OXA-51	OXA-23
3	_	POS	POS
2	_	POS	POS
18	_	POS	POS
7	_	POS	POS
N25	_	POS	POS
N55	_	POS	POS
N5	_	POS	POS
N7	_	POS	POS
M-880 GNR	_	POS	POS
M-224 GNR	_	POS	POS
M-968 GNR	_	POS	POS
M-964 GNR	_	POS	POS
M-933 GNR	_	POS	POS
M-201 GNR	_	POS	POS
M-1095 GNR-S	_	POS	POS
M-221/PP GNR	_	POS	POS
M-1109GNR	_	POS	POS
M-223/PP GNR	_	POS	POS
M-1223 GNR	_	POS	POS
4818	_	POS	POS

DISCUSSION

The pathogen Acinetobacter has become a major concern and difficulty for the healthcare system [4,50]. The rise of multidrug-resistant Acinetobacter isolates has emerged as one of the most significant concerns in hospital settings, notwithstanding the severity of infections [51]. The last line of defense against MDR A. baumannii was discovered to be carbapenems [52, 53]. However, several nations, including China, India, Turkey, Greece, Italy, Spain, Pakistan, Germany, and England, have begun to face a common threat from the increasing incidence of carbapenem resistance [19]. However, whereas the prevalence of MDR A. baumannii has been widely reported [4], there's very little information available about CRAB from Pakistan in prior studies. The frail healthcare system in Pakistan is finding it difficult to treat infections brought on by CRAB, that has led to higher mortality and morbidity rates [5].

In this study, within the 48 strains of Acinetobacter baumannii, 46 strains showed carbapenem resistance while 2 (4.16%) were carbapenem-sensitive strains. The OXA51 gene was spotted in forty-five carbapenem-resistant Acenitobacter, while 1/2 carbapenem-sensitive strains carried the OXA51-like gene. The most prevalent carbapenem hydrolyzing -lactamase genes in A. baumannii, however, were OXA-51-like and OXA-23-like. Moreover, in present study, the most prevalent form of resistance in A. baumannii is thought to be produced by the oxacillinase enzymes.

Furthermore, in a previous study from Pakistan [56], the prevalence of the blaOXA-23 and blaOXA-51 genes in A. baumannii was reported to be 90 percent and 70 percent, respectively. Another study found that the presence of bla-OXA-51-like was present in all 39 isolates with carbapenem resistance and that blaOXA-23-like was present in 97.4 percent of isolates [30].

In our investigation, A. baumannii strains carrying the blaOXA-23 and blaOXA-51 genes had a high rate of carbapenem resistance, including imipenem (95.83 percent) and meropenem (97.91 percent). Whilst in contrast, data previously recorded and published in 2021 revealed that isolates of A. baumannii exhibited a carbapenem-resistant rate of 64.9% [50]. In addition, all carbapenem-resistant strains (95.83%) also showed 100% resistance to cefepime and amikacin. While resistance was also shown to other antimicrobials, including 97.91% to AMP, 87.50% to SXT, 85.42% to TIC, 83.33% to CIP, 83.33% to TE, 72.92% to CN, 56.25% to DO,

58.33% to TG, respectively. However, comparatively, susceptibility was shown 35.41% towards tigecycline, sulfamethoxazole/trimethoprim 12.50%, ticarcillin 14.50%, gentamicin 16.66%, ciprofloxacin 6.25%, doxycycline 35.41%, and tetracycline 6.25%.

In addition, all 48 strains were MDR due to the mutations and different mechanisms of carbapenem resistance, thus leaving few therapeutical options. Moreover, strains of Acinetobacter resistant to IMP were phenotypically tested by CDT for Metallo-beta-lactamase production.

Out of 48 strains of MDR *Acinetobacter baumannii,* 91.64% showed positive results for MBL. Although just 2.1 percent of carbapenem-resistant Acinetobacter species MBL positive results were seen in the past [4]. The generation of MBL in A. baumannii has also been documented in numerous investigations conducted worldwide [54,55].

Moreover, New Delhi Metallo-beta-lactamase is the latest class B β-lactamases that have been reported in many countries throughout the world. In contrast to a previous investigation from Pakistan, where the blaNDM-1 gene was found in only one CRAB isolate, the current study found the blaNDM gene in four strains [16]. This shows that the blaNDM gene is becoming more prevalent in A. baumannii. Most NDM-1carring strains were found to be resistant to the effects of meropenem, amikacin, imipenem, cefepime, trimethoprim-sulfamethoxazole, tetracycline while the highest susceptibility was observed for tigecycline. A study from Nepal found that the blaNDM-1 and blaOXA-23 genes were 13.6 percent concordant in A. baumannii [58]. While in this study, all blaNDM positive isolates (8.33%) carried the OXA-23-like gene. However, only one blaNDM harbouring carbapenem-resistant strain of A. baumannii lacked the OXA-51-like gene.

Additionally, in a previous study from Pakistan, the oxacillinase 23 gene was discovered in conjunction with the oxacillinase 51 gene [57]. However, 2.17 percent of strains were found to have the OXA-23-like gene independently present in this study. These variations expose that different CR-AB isolates have different levels of resistance genes. Additionally, these results demonstrate that isolates are becoming more and more resistant, which can have disastrous treatment consequences. There is currently a lack of information on the percentage of Pakistani MBL and carbapenem resistance, which could result in the development of the new Delhi metallo beta-latamase. Therefore, it is vital to control AMR and the spread of the NDM gene.

CONCLUSION

Currently, little is known about the existence of the blaNDM gene in Pakistani A. baumannii, which is resistant to carbapenem (CR-AB). This study examines the incidence of class D (OXA-51-like, OXA-23-like) and class B (blaNDM) carbapenemases and variations of antibiotic sensitivity in CRAB isolates from several acute care facilities across Rawalpindi and Islamabad, Pakistan. In the present study, the high presence of multidrug-resistant Acinetobacter Baumanni in hospitals including AFIP, CITI lab, and NIH from Rawalpindi and Islamabad, were detected. All strains were carbapenem resistant, carring OXA-23-like, blaNDM-1, and OXA-51-like genes. The results highlight the necessity of screening in hospital and community, at the national level in Pakistan in order to assess the precise incidence of carbapenemase-producing A. baumannii and to halt the spread of infections that are blaNDM-carrying.

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