ORIGINAL ARTICLE

Incidence, Characterization and Anti-Microbial Susceptibility Pattern of Pseudomonas aeruginosa Isolated from Clinical Subjects

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ABSTRACT

Background: The increasing resistance of Pseudomonas aeruginosa to many antimicrobials poses a major therapeutic challenge and this is of great concern in hospitals due to rise in nosocomial infections.

Objectives: To identify the prevalence of Pseudomonas aeruginosa and to compare the antimicrobial activity of antibiotics alone and in combination with $AgNO_3$ nanoparticles against multidrug resistant Pseudomonas aeruginosa.

Methods: Study was conducted at Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore from July 2018 till March 2019. Total 1800 samples were collected from admitted patients in a tertiary care hospital. The isolates were identified by colony morphology and different biochemical tests and confirmed by Analytical Profile Index (API). Antibiotic susceptibility was determined by disc diffusion method.

Results: Out of 1800 samples, 90 were of Pseudomonas aeruginosa. Prevalence of Pseudomonas aeruginosa was only 5%. Antibiotic sensitivity of isolated Pseudomonas aeruginosa showed best zones of inhibition against Colistin. Among the 90 isolated strains, 88% showed sensitive zones to colistin and only 12% were resistant. 67% isolates were sensitive and 13% showed intermediate sensitivity to Tazobactam/Piperacillin. 65%isolates were sensitive to Cefepime and 63% were sensitive to Ceftazidime. 47% and 49% strains were sensitive to aminoglycosides gentamycin and amikacin respectively. 67% isolated were susceptible to Tobramycin. 52% showed resistance to imipenem and 62% were resistant meropenem.

Conclusion: The current study revealed only 5% Prevalence of Pseudomonas aeruginosaand highest resistance against meropenem.

Key words: Pseudomonas aeruginosa, Nosocomial pathogen, Carbapenem-resistant Pseudomonas aeruginosa

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacteria that can be separated from soil, water, plants, animals, and human. Human colonization occurs generally at wet areas, for instance, perineum, axilla, and ear. Pseudomonas aeruginosa among various pathogens can be found in the subungual areas of hands as well as non-living surfaces such as respiratory treatment equipment, endoscopes, contaminated resting pads, disinfectants, corrupted water supplies, IV plans, and in pools used for practice based recovery or hydrotherapy.[1]

Countless therapeutic methods are employed each year worldwide yet the treatment of the postoperative wounds is becoming challenging because of the development of multidrug resistance.[1]Pseudomonas aeruginosa is a gram-negative pathogen which can cause perilous maladies in hospital admitted patients. Multidrug resistance makes infections caused by Pseudomonas aeruginosa much difficult to treatespecially in patients who are suffering with wounds, previous lung infections like cystic fibrosis. As reported by Center for Disease Control, more than 51,000 clinical defilements are represented each year in the USA with 400 deaths every year. Pseudomonas aeruginosa has multiple tools that make it resistant to commonly used antimicrobial agents. Various resistance mechanisms make it multidrug resistant elevating morbidity and mortality rate.[2]

The association of multiple resistance mechanisms prompts the need to search for alternative treatment modalities. These Biofilms make a safer haven for such microbes.[3]These biofilms decrease the antimicrobial passage ways plusprovide protection from antibiotics.[4]Cytotoxic phenotypes start festering inside biofilm arrangements due to strong phospholipase development.[5]

Among medically important bacteria, Vibrio, Burkholderia, Leptospira and Brucella species are those with two chromosomes, while Borrelia burgdorferi has its genome in a quick chromosome.[6]The enormous model in bacterial genome size is that, everything considered, free-living species have more prominent genomes than parasitic species which along these lines have more noteworthy genomes than many pathogens. Bacterial genomes fluctuate in a general sense between animal varieties as for nucleotide affiliation: The G+C (guanosine-cytosine) substance may change locally inside a genome, at any rate it is sensibly uniform inside a bacterial grouping or animal classes, connecting from around 25% in Mycoplasma spp. to around 75% in some Micrococcus species.[6]The extending utilization of nanoparticles have brought new perspectives towards new antimicrobial materials. Nano-composites can be the solution to problems posed by multidrug resistance Silver nanoparticles (AgNPs) show exceptional properties. Their usage is broad as they are conveyed in different ways depending upon the sort of material and its applications.

Nanoparticles are among the most outstanding segments in science and nanotechnology today.[7]Silver is used as a powerful agent against fungi, bacteria and viruses with a wide range of antimicrobial activities. AgNPs

have been considered to have strong antibacterial effects. AgNPs are typically less than 100 nm in size with 20–15,000 silver atoms and unusual physical, compound and biological properties.

MATERIALS AND METHODS

Chemicals used in lab: Constituents of media were attained by the following: E. Merck (Darmstadt, Germany), Difco Labs (Detroit Michigan, USA), Scharlau laboratories (France), Panreac Quimica (Barcelona, Spain) and Sigma-Aldrich Chemicals Co. (St. Louis, USA).

Requirements of laboratory apparatus: The laboratory instruments required for the procedure were as follows-Sterile sample container, Sterile cotton swabs, Petri plates (25ml), Flask (200ml-500ml), Beaker (200ml-500ml), Test tubes (20ml), Glass slides, Sterile syringes, Micropipettes, Sterile tips, Eppendorf tubes, Inoculation platinum wire loop, Bunsen burner, Incubator (temp. range 35-37ºC), Hot air oven, Water bath, Autoclave (121ºC, 15psi), Vortex mixer, Weighing balance, Colony counter, Compound microscope, Glass test tubes holder, Glass slides, Laminar flow, Biosafety cabinet level 2, Digital weighing balance.

Media and chemicals required: The media and chemicals required for the procedure were-Nutrient agar, Differential media (Cystine Lactose Electrolyte Deficient agar CLED), Antibiotics specific media (Mueller Hinton Agar), 70% and 95% ethanol, Distilled water, Normal saline, Glycerol, Kovac's reagent, Simmons citrate agar, Triple sugar iron agar with indicator, TMPD dihydrochloride, Urease broth base agar, Urea, Distilled water, Gram staining reagents, Standard antibiotics and Silver nanoparticles ($AgNO_3$).

Antibiotics used in research work: Antibiotics used in study werecolistin, piperacillin/tazobactam, cefepime, ceftazidime, gentamycin, amikacin, tobramycin, imipenem and meropenem.

Primer information: All material needed for sequencing reaction was supplied to Macrogen sequencing company (Seoul, South Korea). Macrogen sequencing company used ABI 3730X1 DNA sequence (applied biosystem, USA) and the nucleotide sequencing was done by Sanger sequencing technique. Eight reactions were done by this method.For sequencing primer used
785F5 (GGA TTA GAT ACC CTG GTA)3´ and 907R´(CCG TCA ATT CMT TTR AGT TT)3´ for PCR primer sequences are 27F5´(AGA GTT TGA TCM TGG CTC AG)3 and 1492R5´(TAC GGY TAC CTT GTT ACG ACT T)3´.

Collection of samples: A total of 1800 clinical samples were collected from different wards in tertiary care hospitals in Lahore. These samples were obtained aseptically to avoid contamination. These were labelled properly and transported to the lab safely.

Antibiotics sensitivity testing: Antimicrobial susceptibility was determined by disc diffusion method. Mueller-Hinton agar plates were organized and bacterial suspension prepared according to McFarlands standard. Antibiotic discs were applied and the discs incubated for 24hrs. Zone of inhibition was measured and noted.

Procedure for DNA extraction:10ml of distilled water was taken in a test tube. Weighed 0.4g of nutrient broth was added to the test tube containing distilled water. These test tubes were autoclaved for 15min at 121ºC. Following sterilization, the media was allowed to cool. This was pouredin the store culture (Eppendorf) tubes and incubated overnight. 1ml bacterial culture was taken in Eppendorf tube and centrifuged for 7000rpm for 5min. Following centrifugation, the supernatant was separated. TE buffer 400µl and 100µl SDS 10% added followed by 5µl protease K and incubated at 37ºC for one hour. 500µl phenol chloroform mixer was added at room temperature and centrifuged at 10,000rpm for 10min. Three pellets formed-aqueous pellet, inter pellet and organic pellet. Aqueous pellet was transferred to fresh Eppendorf tube for next process. 500µl potassium acetate or sodium chloride at pH of 5.2 was added and 1ml of isopropanol added. Centrifuged at 10,000rpm for 10min. The supernatant was discarded and pellet saved for next process. Washed with 70% ethanol and centrifuged at 5,000 for 5min. This was followed by adding25µl injection water in each eppendorf.

Molecular characterization: This was done with the help of primers and polymerase chain reaction PCR mentioned in previous section.

Sequencing of DNA:BLAST software was employed for DNA sequencing of Pseudomonas aeruginosa as depicted in Figures 3 & 4.

RESULTS

Ninety were positive for Pseudomonas aeruginosa. The prevalence of Pseudomonas aeruginosa in clinical specimens was only 5%. Among the positive cultures, 55%were of females while 35% were of males. Only 9(1%) patients were below 1 year of age, 9(10%) patients were between 1 to 18 years, 55(61%) patients were between 19 to 50 years and 25(28%) patients were above 50 years of age. Clinical specimens were 48(53%) from pus/wound swabs, 6(7%) blood, 11(12%) urine and 25(28%) high vaginal swab (HVS).

The distribution among the specimens received from different departments included ICU (4%), neonatal nursery (0%), medicine ward (9%), surgery (21%), obstetrics and gynaecology (8%), paediatrics (1%) and outpatient department (57%) as shown in figure 5.The isolated Pseudomonas aeruginosa showed highest sensitivity to colistin. Among the 90 isolates, 90% showed sensitive zones to colistin and only 10% were resistant. 67% were sensitive and 13% showed intermediate sensitivity to tazobactam/piperacillin. 65% isolates were sensitive to cefepime. 63% showedsensitivity to ceftazidime. 47% and 49% strains were sensitive to aminoglycosides i.e gentamycin and amikacin respectively. 67% of the isolates were sensitive to tobramycin. 52% and 62% isolates were resistant to imipenem and meropenem respectively. These results are shown in figure 1. In the current study, all the isolates of Pseudomonas aeruginosaexhibited multidrug resistance i.e. they were resistant to ≥4 classes of antimicrobial drugs.

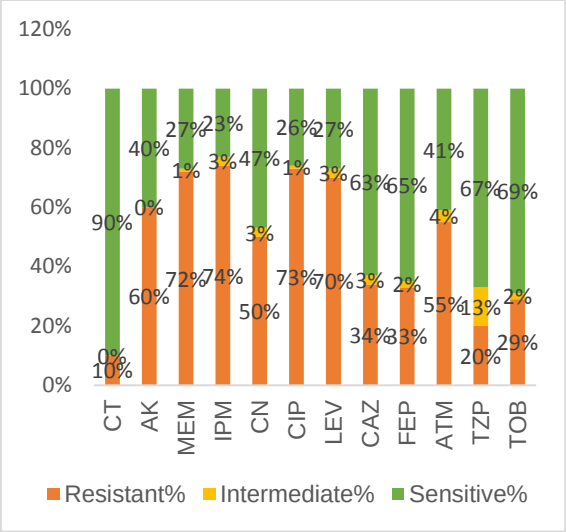

Fig. 1: Results of Antibiotic Sensitivity testing against antibiotics

Table1: Zone of Inhibition of AgNPs with different concentrations against Pseudomonas aeruginosa

AgNPs Concentration	Zone of Inhibition of Pseudomonas aeruginosa
10ul	10mm
20ul	14mm
30ul	16mm
40ul	18mm
50ul	20mm

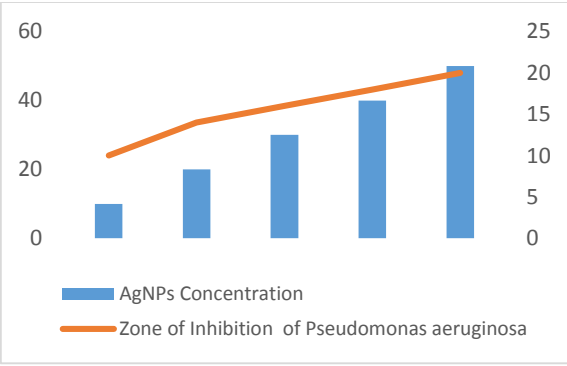

Fig. 2: Zone of Inhibition of AgNPs with different concentrations against Pseudomonas aeruginosa

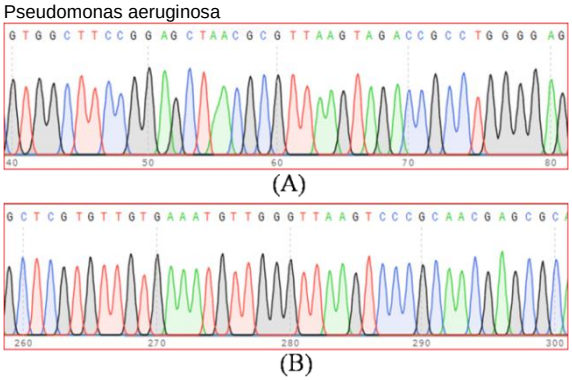

Fig. 3:A and B. Trace file analysis. In which different peak showed variation. Black peak describe the value of Guanine, Pink describe cytosine, Red describe thiamine and Green describe adenine. A trace files analysis shows result of 40 to 80 nucleotides peak of different colors which indicate different pattern. In figure B trace file analysis shows 260 to 300 nucleotides sequence peak which represent different level.

CCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAG
TAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC
TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGA
TTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCA
GCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGT
GATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA
TCATGGCCCTTACGACCAGGGCTACACACGTGCTAC
AATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGC
AAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTG
GAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTA
GTAATCGTGGATCAGAATGCCACGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGG
GGGGCGCTTACCACTTTGTGATTCATGACTGGGGT

Fig. 4: Blast result showed gene sequence of isolated species of Pseudomonas aeruginosa

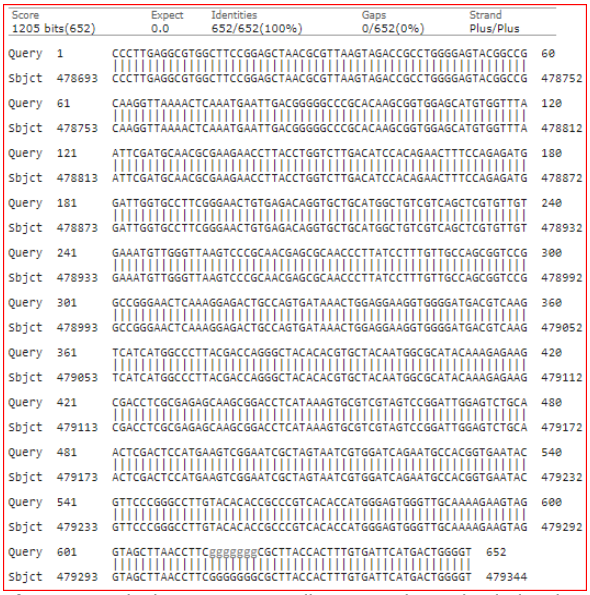

Fig. 5: A pairwise sequence alignment showed relation between Query and Subject.

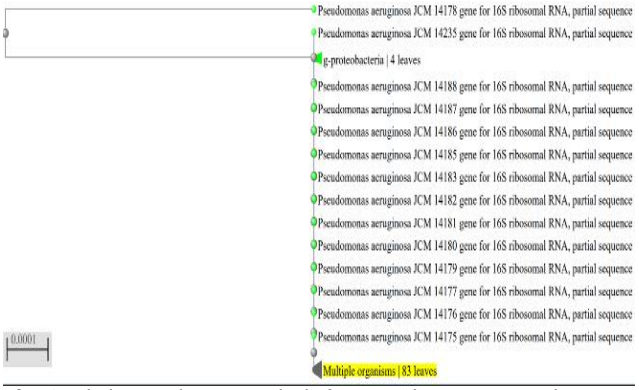

Fig. 6: Phylogenetic tree analysis for Pseudomonas aeruginosa

DISCUSSION

The quality of 16SrRNA is used for phylogenetic examinations since it is observed significantly between different types of minute living beings and archaea. Given these, Carl Woese introduced use of 16S rRNA, which is further intensified by mitochondrial and chloroplastic rRNA. 16S rRNA consistency action courses include hypervariable districts that can provide help for bacterial distinctive confirmation by species-unambiguous imprint groupings.16S rRNA quality sequencing has become inevitable as a quick and unassuming alternative in remedial microbiology rather than phenotypic strategies for distinctive bacterial evidence, as needed. Additionally, it has been used to delineate new species that were never successfully refined as has been done for the isolates of Pseudomonas aeruginosa as shown in figures 5 and 6.

The purpose behind this study was to isolate Pseudomonas aeruginosa specimens from infected patients and to detect the multidrug resistance against commonly employed antibiotics used in clinical practice. From 1800 specimens, only 90 were found to be Pseudomonas aeruginosa. Higher isolation levels have been reported with Pseudomonas aeroginosa from respiratory samples in other countries.[8]Pseudomonas aeruginosa resistance levels against amikacin, gentamicin, ceftazidime, ciprofloxacin, piperacillin/tazobactam and to imipenem as recorded in a surveillance sample of five European countries intensive care units (ICU) is lower.[9]Pseudomonas aeruginosa strains from 11 European countries were confirmed to be susceptible to imipenem and meropenem between 1997 and 2000, with resistance levels of 16% and 18% respectively.[10]

Results of this study have showed all the strains to be gram negative and non-motile when grown on nutrient gar and other selective/ differential media. These isolates had the capacity to produce acid from glucose and lactose. All strains showed positive catalase and oxidative fermentation. The negative reactions were acid production from sucrose, H_2S on TSI and gas production, mannitol, indole and oxidase tests. Pseudomonas aeruginosa is an important nosocomial pathogen in hospitalized patients causing high morbidity and mortality. With the growth of antibiotic resistance over the past few years, Pseudomonas aeruginosa poses a serious medical concern.

It has emerged as a leading cause of nosocomial infections, especially in intensive care units (ICUs), causing a range of infections including septicemia, infections of the urinary tract and infections of wound.[11]Multidrug resistant Pseudomonas aeruginosahas been thecause of diseases and outbreaks in the past twenty years and these outbreaks have been recorded globally, thus solving this issue of multidrug resistance in Pseudomonas aeruginosais critical.[12, 13]

Research based on Pseudomonas aeruginosa from twelve countries indicate a drastic increase in resistance levels against imipenem and meropenem. Various reports have shown that resistance to imipenem has increased from 0-40% from 2000 to 2004.[14]

As ccording to various reports, imipenem resistance in Pseudomonas aeruginosa isolates has risen from nil in 1991 to much peaked in 2001.[15]Resistance against ampicillin/sulbactam, imipenem, and meropenem are 51.6%, 26.3%, and 29.6%, respectively.[16]

CONCLUSION

Thus it is imperative to find alternate solutions to antibiotic treatment regimes so as to encounter the rising problem of multidrug resistance in Pseudomonas aeruginosa.

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