

Computational and Molecular Studies Related to Multidrug Resistant Microorganisms Isolated from Clinical Sample

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Abstract

To evaluate the bacteria belonging to genus *Pseudomonas* sp, *Staphylococcus* sp *E.coli*, *Klebsiella* sp obtained from various clinical Samples (urine, blood, pus, and catheter) obtained from diagnostics labs and hospitals of Madurai were screened for their multi drug resistance using standard antibiotics such as Ampicillin trihydrate (A), Chloramphenicol (C), Gentamycin (G), Tetracycline hydrochloride (T). Ampicillin (A), Ceftazidime(CA), Amikacin(AK), Levofloxacin(LE), Cefuroxime(CU), Cotrimoxazole(CO), Nalidixic acid (NA), Streptomycin (S), antibiotics with the National committee for clinical laboratory standard (NCCLS) new interpretive criteria. The isolates that were showing multi drug- resistance were further evaluated for polymerase chain reaction PCR as a gold standard method to evaluate the resistant gene by the method, Muller Hinton agar (disc diffusion method). The results showing Multidrug resistant gene that has as vehicles for transmission of genetic information involved in spread of antibiotic resistance into various species, and to analyze the gene possessing the features of MDR using PCR amplification of suspected genes associated with resistance production.

1. Introduction

The term "Antibiotic" refers to any agent that has the biological activity against living organism, which was originally developed to treat human infectious diseases. The antibiotics can be classified as either bactericidal or bacteriostatic. Bactericidal kills the bacteria directly, where the bacteriostatics prevents the bacteria from dividing. The broad use of antibiotics had created a strong selective pressure, which consistently had resulted in the survival and spread of resistance that has evolved with the increased number, volume and diversity of antimicrobial applications. (Baquero *et al.*, 2003)

Bacteria are able to inherit antibiotic resistant genes to provide protection against most antibiotics. The dissemination of antibiotic genes by horizontal gene transfer has led to the rapid emergence of antibiotic resistance among bacteria (Barlow *et al.*, 2004). The rapid dissemination of drug-resistant bacteria is an alarming and increasing problem, complicating the treatment of infections. Much of the problem is the result of antibiotic resistant genes transferring among bacterial species, carried by plasmids and transposons (Stokes *et al.*, 1989).

Multi- drug resistance bacteria need new approaches to the management and treatment of infections in hospitalized patients. According to them the health care associated infection cause substantial resistance and which requires a multidisciplinary approach and closer collaboration among health care members in hospitals,

pharmacist, infection control practitioners and infectious disease specialists. They can reduce the treatment failures and minimize the spread of multidrug- resistance organisms between the hospital environment and the community.

The emergence of antimicrobial resistance pathogens now treats the discovery of potent antimicrobial agents. Antimicrobial resistance has resulted in increased morbidity and mortality as well as health care costs. Yearly expenditures arising from drug resistance in the United States are \$4 billion and are rising (Archibald *et al.*, 2007).

The tremendous therapeutic advantage afforded by antibiotics is being treated by the emergence of increasing the resistance strains of microbes. Selective pressure favoring resistant strains arises from misuse and over use of antibiotics (Thomas *et al.*, 1999).

The emergence of antibiotic resistance is an evolutionary process that is based on selection for organism that has enhanced ability to survive. Dose of antibiotic that would previously be lethal (Cowntey *et al.*, 2008).

The evolution of antibiotic resistance in bacteria is a topic of major medical importance. Evolution is the result of natural selection ridding on variant phenotypes. Both the rigid base sequence of DNA and the more plastic expression patterns of the genes present define phenotype. Evolution requires phenotypic variation, selection

and heritability. It is generally assumed that mutation provides the single source of biological diversity that fuels evolution (Motce et al., 2008)

The change in the genetic composition of an organism may occur by 1 or 2 mechanisms there may be a acquisition of new genetic material mutations are generally uncommon events, perhaps occurring at a frequency of 1 event per $10^7 - 10^{10}$ bacteria, but may result in the development of resistant during therapy in organisms that are initially susceptible (Michael et al., 2009).

This work broadly gives knowledge about the role of Multidrug resistant gene that has as vehicles for transmission of genetic information involved in spread of antibiotic resistance into various species, and to analyze the gene possessing the features of MDR using PCR amplification of suspected genes associated with resistance production.

2. Materials and Methods

Collection of clinical isolates

Clinical isolates were obtained from meenakshi mission hospital, Madurai and Bose Clinical laboratory, Madurai.

Clinical samples used

Blood, Pus, Catheter, Urine etc from UTI (Urinary Tract infection) patients.

Glassware and media sterilization

The glassware were washed thoroughly in running tap water, rinsed in distilled water and dried in a hot air oven (Yarco hot air sterilizer 1-431 max temp-200°C (New Delhi). Media were sterilized in a portable autoclave (Equitron, Mumbai) at 121°C and at 15 lbs pressure for 15 minutes. All the incubation works were carried out under aseptic condition in a Laminar Air flow chamber (Atlantis – clear air). Incubation of all culture was kept in an incubator (NSW – 150 max temp- 60°C, New Delhi).

Processing of samples

About 100 Clinical samples were collected from UTI patients over a 3-month period. The strains were isolated and plated. The isolated bacteria were collected on tryptone agar slants, and were purified twice on MacConkey's agar. They were later maintained on tryptone agar slants. Most bacteria were already identified at source, however if needed, identification was carried out by biochemical reactions and by the reaction on T.S.I medium. The Bacterial isolates obtained were belonging to the groups *E.coli*, *Pseudomonas sp*, *Klebsiella sp*, *Staphylococcus sp*.

Antibiotics

The antibiotics used were: Ampicillin trihydrate (A), Chloramphenicol (C), Gentamycin (G), Tetracycline hydrochloride (T), Ampicillin (A), Ceftazidime(CA), Amikacin (AK) Levofloxacin (LE), Cefuroxime(CU), Co-trimoxazole(CO), Nalidixic acid (NA), Streptomycin (S), (May and Baker, Nigeria). The control strains were run simultaneously with the test organisms. Results were interpreted with the National Committee for Clinical Laboratory Standards now known as Clinical Laboratory Standard Institute (CLSI) criteria for disk diffusion (NCCLS, 2000). All the antibiotics were obtained from Sigma Chemical Company, U.S.A.

Preservation of isolates

Glycerol stocks were prepared and stored at -80°C for long term preservation. Pure cultures strains were incubated at 50°C for 48 h in isolation broth. Then 0.5 ml of each of the cultures were transferred into cryotubes and 0.5 ml broth containing 40% glycerol was added. The samples were mixed gently and stored at -80°C.

Susceptibility testing

Antibiotic susceptibility (of both the clinical isolates and the *Escherichia coli* transconjugants) was calculated by the disk diffusion method on Mueller-Hinton (MH) agar according to NCCLS (National Committee for Clinical Laboratory Standards, USA) recommendations. Antimicrobial agents tested were Ampicillin trihydrate (A), Chloramphenicol(C), Gentamycin(G),Tetracycline hydrochloride (T), Ceftazidime(CA),, Amikacin (AK), Levofloxacin (LE), Cefuroxime (CU),Co-trimoxazole(CO), Nalidixic acid (NA), Streptomycin (S), (May and Baker, Nigeria). The minimum inhibitory concentration (MIC) of selected aminoglycosides was then determined by the agar dilution technique on MH agar plates with an inoculum of 10⁴ CFU per spot according to NCCLS recommendations.

Curing of plasmids

For studying curing by acridine orange, the bacterium bearing R plasmid was grown overnight in L.B. broth. To the fresh L.B. broth was prepared containing 120 µg/ml of acridine orange was inoculated with the overnight culture to give a 100 fold dilution. All this work was carried out in the dark because of the light sensitivity of acridine orange. A control tube lacking acridine orange was always included. The test & the control cultures were incubated overnight at 37°C. The test culture & the control culture were then diluted & plated on MacConkey's Agar plates, to obtain isolated colonies. The colonies from each plating were

grided onto MacConkey's Agar plates and Nutrient agar plates. After overnight incubation at 37°C, these were replicated on antibiotic containing plates to check for the loss (or its absence) of antibiotic resistance determinants. Plasmid DNA were extracted by the method (Kado and Liu), Subjected to Electrophoresis through agarose 0.8% gel, stained with ethidium bromide, photographed under UV transilluminator.

Transformation of resistant plasmid

Extraction of Total DNA of E. coli was extracted using Genomic DNA Extraction kit (Promega, USA) following manufacture's protocol for Gram-negative bacteria. The PCR solution was composed of 10 × buffer 2 µl, dNTPs (2.5 mM) 0.4 µl, 5'CS/3'CS (10 pmol/ µl) 0.5 µl each, Taq DNA polymerase (5 U/ µl, Promega, USA) 0.2 µl, distilled water 15.4 µl and template DNA (50 ng/ µl) 1 µl. Amplification consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30s, and extension at 72°C for 2 min 30s and a final extension at 72°C for 5 min. Amplicons were analyzed through electrophoresis on 1.0% agarose gels, and 1 kb ladder (Takara, Japan) was used as a molecular size marker.

3. Results

The clinical isolates were collected from various clinical Samples (urine, blood, pus, and catheter) obtained from diagnostics labs and hospitals of Madurai, processed and characterized for the characterization of Morphological, biochemical characters of the isolates, the results evaluated belonging to the genus *Pseudomonas sp*, *Staphylococcus sp*, *E.coli*, *Klebsiella sp*. The isolates were further identified for the resistance pattern by performing antibiotics susceptibility test, disc diffusion method using standard antibiotics such as Ampicillin trihydrate (A), Chloramphenicol (C), Gentamycin (G), Tetracycline hydrochloride (T). Ampicillin (A), Ceftazidime (CA), Amikacin (AK) Levofloxacin (LE), Cefuroxime (CU), Co-trimoxazole (CO), Nalidixic acid (NA), Streptomycin (S), using control organisms and antibiotics with the National committee for clinical laboratory standard (NCCLS) new interpretive criteria. The results showing Multidrug resistant gene that has as vehicles for transmission of genetic information involved in spread of antibiotic

resistance into various species, and to analyze the gene possessing the features of MDR using PCR amplification of suspected genes associated with resistance production.

Fig: 1 *Pseudomonas sp*

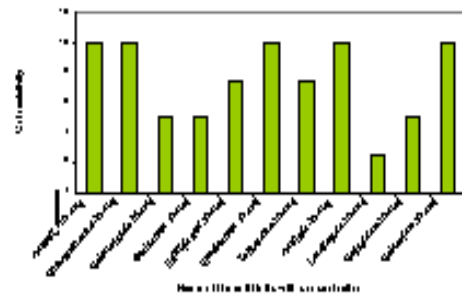


Fig: 2 *E.coli*

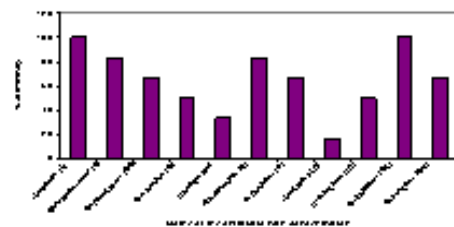


Fig: 3 *Staphylococcus sp*

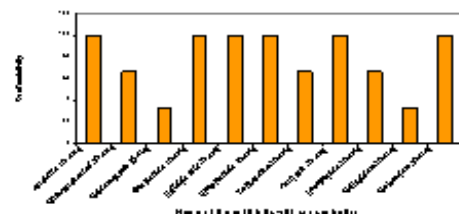
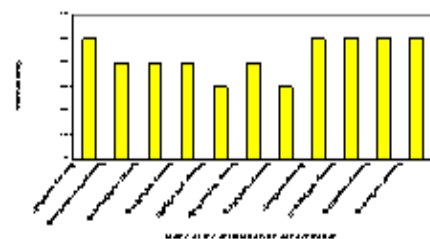


Fig: 4 *Klebsiella sp*



Graph: I Antibiotic sensitive / resistance pattern of the clinical isolates and their percentage of resistance for *Klebsiella sp*, *Pseudomonas sp*, *E.coli*, *Staphylococcus sp*

| Organisms | Antibiotics | | | Zone of inhibition (mm) | | | | | | | | |
|-----------------------|-------------|---|----|-------------------------|----|----|----|----|---|---|---|--|
| | T | C | CA | LE | CU | Co | NA | Ak | S | G | A | |
| <i>E. coli</i> | R | I | R | R | R | R | R | R | R | R | R | |
| <i>Staphylococcus</i> | R | I | R | R | R | R | R | R | R | R | R | |
| <i>Pseudomonas</i> | R | R | R | R | R | R | R | R | R | R | R | |
| <i>Klebsiella</i> | R | I | R | R | R | R | R | R | R | R | R | |

Antibiotic Suceptibility test (UV mutation)

| Organisms | Antibiotics | | | Zone of inhibition (mm) | | | | | | | | |
|-----------------------|-------------|---|----|-------------------------|----|----|----|----|---|---|---|--|
| | T | C | CA | LE | CU | Co | NA | Ak | S | G | A | |
| <i>E. coli</i> | R NZ | I | R | R | R | R | R | R | R | R | R | |
| <i>Staphylococcus</i> | I | S | R | R | R | R | R | R | R | R | R | |
| <i>Pseudomonas</i> | R | R | R | R | R | R | R | R | R | R | R | |
| <i>Klebsiella</i> | R | R | R | R | R | R | R | R | R | R | R | |

Antibiotic Suceptibility test (Normal strains)

Plate: a *E.coli*

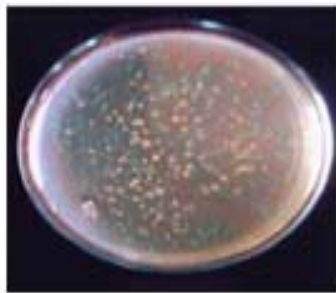


Plate: b *Pseudomonas sp*

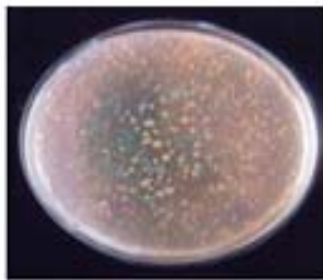
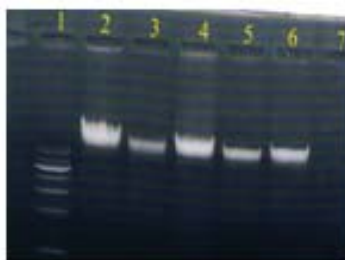


Fig: a Agarose gel



Transformation: Blue White Selection- Host cell: *E.coli* DH5 α ; Resistant Plasmids Plate: a and

Plate: b. Fig a: Agarose gel: 0.8% Showing results of Plasmid DNA for isolates

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