Molecular Characterization and Phylogenetic Analysis of Multidrug Resistant Microorganism with Special Reference to Nosocomial Infections

Khajuria Komal, Sharma Neha* and Tomar Rajesh Singh
Amity University Madhya Pradesh, Maharaj Pura Dang, Gwalior (M.P.), INDIA
*nsharma2@gwa.amity.edu

Abstract

The bacterial isolates from hospital environment act as a pool of antimicrobial resistance as well as main cause of nosocomial infections and their genes act a potential source of resistance to clinical pathogens. In the present study, we aim to perform molecular characterization of bacteria from hospital settings and to identify the emerging multi-drug resistant bacteria in our study area. A study was carried out in various hospitals in Gwalior, Madhya Pradesh, India. Collection of isolates was carried out, transported and immediately analyzed by using standard protocols. Identification of bacterial isolates was done using biochemical tests and 16S rDNA sequencing technique. Mueller-Hinton agar was used for carrying out antibiotic susceptibility test against beta-lactam group of antibiotics using Kirby-Bauer disk diffusion method.

Amplification of DNA was done was using polymerase chain reaction with the help of universal primers. A total of 46 bacterial isolates were found from different areas of various hospitals. Four isolates were picked for molecular characterization and phylogenetic tree construction using distance matrix method. We found that three out of four belong to Staphylococii group whereas one is from Bacilli group. In conclusion, we have found 4 prominent bacterial strains as MDR against beta-lactam group of antibiotics and it was observed that variations in the sequences of target bacterial species can be attributed to provide resistance to different antibiotics and gene transfer among bacterial strains.

Keywords: Multi-Drug Resistant Bacteria, Nosocomial Infections, Beta-Lactam Antibiotics and Phylogenetic Tree.

Introduction

Multiple drug resistance in bacteria against the various antibiotics possesses a serious global health challenge which ultimately results in increased rate of mortality and morbidity. It is reported by WHO that by 2050, yearly mortality rate will rise to approximately 10 million. Multiple drug resistant bacteria mostly belong to *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter, Pseudomonas aeruginosa* and *Enterobacter* which form ESKAPE GROUP. Multi-drug resistant microorganisms (MDROs) are bacteria which show resistance to one or more than one class of antibiotics. They are mainly belonging to methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* species (VRE), carbapenemase-producing *Enterobacteriaceae (Klebsiella pneumonia*, *Escherichia coli* and *Acinetobacter baumannii)*. These kinds of organisms manufacture chemicals that enable them to resist the impact of certain antibiotics or antimicrobial agents and this adaptation is well passed between totally different species. They are primarily immune to the broad spectrum antimicrobial agents. Due to MDROs' resistances, there is limitation of treatment for patients, creating infection crucial to preventing more harm[1].

Multidrug resistant bacteria are standard organisms and one amongst the important cause is related to public health. Antibiotics helped to save the lives of countless patients. However, the clinical effectiveness of many pre-available antibiotics is becoming defenseless due to emerging problem of MDR (Multi Drug Resistant) microorganism. (MDR)[19]. Extended hospitalization results in many infectious diseases or infections caused by many multi-drug resistant micro-organisms and also results in increased risk of mortality as well as morbidity. In particular, resistance is mainly caused due to improper functioning of immune systems like in cancer patients, AIDS or during transplantation processes. Today's community is mostly dependent on prolonged use of antibiotics and its continuous use causes resistance among majority of human pathogens.

Infections caused by multi-drug resistant bacteria are very sophisticated and are not easy to treat. *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* are good examples of multi-drug resistant microorganisms. These microbes could also be community or hospital acquired[13]. Possible health risk includes dispersal of diseases by the pathogens and extensive dissemination of antimicrobial resistance genes. Hospital waste may be unsafe to community health and ecological balance because it is containing a number of pollutants like chemical and pharmaceutical wastes. Development of resistance in number of human pathogens on large scale results into multidrug resistance which ultimately results in increased rate of global mortality as well as morbidity because we are living in the era of antibiotics[24].

In this pandemic, bacterial strain belonging to ESKAPE group mainly (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter, Pseudomonas aeruginosa* and *Enterobacter*) plays a significant role[17]. These groups of pathogens include both

Gram positive as well as Gram negative bacteria that generally carry genes that determine genetic island of multiple drug resistance gene of complex evolutionary origin that are encoded either by chromosome or plasmids[6,23].

Hospital is a common place that is often used by the individuals and the waste generated at hospital has highest incorporation to provide health and environmental hazards than the wastes of different places[1]. Hospital nosocomial infection is referred to as health facility infection. It is linked with the patient who is admitted for a reason apart from that microbial infection. Nosocomial pathogens are organisms causing conditions that are acquired from the hospital and healthcare climate within limited days of admission and are accountable for nosocomial infections[18]. The pathogens may be associated with inhabited patient either through direct contact or aerosols droplets or stool present in environmental surfaces. These microbial infections may be spread by the tending staff or patients.

Excessive usage of antibiotic is destructive to human health, ecosystem and environment. It could also increase the incidences of drug-resistant pathogens[11]. Antibiotics resistance is a worldwide major problem which is rapidly increasing in both hospitals and the community involved in morbidity, mortality and health-care[14]. Almost in all pathogenic bacteria, it has been observed that microbes are able to obtain the resistance factor to the antimicrobial drugs quickly; therefore, multiple drug resistant bacteria caused the main stoppage in the treatment of infectious diseases[13]. The present study is focused on molecular and phylogenetic analysis of the MDR bacteria based on their antibiotic resistance pattern against beta-lactam group of antibiotics.

Material and Methods

Study area: Gwalior city is within the latitude and longitude, 26.2124° N, 78.1772° E respectively. Gwalior is a city in Madhya Pradesh, India. The bacterial isolated was taken from hospitals within Gwalior or nearby Gwalior regions.

Study design: The study design includes isolation of isolates, biochemical test, antibiotic susceptibility pattern, molecular characterization including Sanger sequencing and phylogenetic tree analysis using distance matrix method. The bacterial isolation and characterization both biochemical and molecular were done at Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior, MP. Sequencing was done at Biokart India Pvt. Ltd., Bangalore.

Collection of samples: Samples were collected from different sections of various hospitals at different hours of activities which include maternity section, surgical wards, washroom area, testing labs, common hall area. The samples were collected in sterile nutrient agar Petri plates and were immediately transported to the microbiology lab within a minimum hour of collection. Samples plates were kept in coolers that contain ice packs and were immediately transported to the lab for further analysis.

Isolation and identification of isolates: The isolates were isolated using method of pour plate[9] and serial dilutions were made according to standard protocols. Aliquots of 1 ml from 10^{-7} and 10^{-8} were spread on Petri plates containing different types of media. All media used in study were prepared according to standard protocols. Each bacterial sample plate was incubated at 37^{0c} for 24 hrs. Sub- culturing was done after incubation based on morphological characters of individual colony and after that streaked on various differential and selective media to get pure cultures e.g. mannitol salt agar (MSA) for *Staphylococcus aureus*, MacConkey agar as a selective agent for *Enterobacteriacea* and eosin methylene blue (EMB) as a selective media for *Escherichia coli*. and then finally stored on nutrient agar media (NAM) plates. Gram staining was done after obtaining pure isolates and was microscopically examined. The biochemical tests include catalase, citrate, indole, methyl red-voges proskauer and sugar fermentation tests[3,20,21].

Antibiotic-susceptibility test: The antimicrobial susceptibility pattern of bacterial isolates was carried out using the disk diffusion method suggested by Kirby-Bauer. The suspension of the bacterial inoculums was prepared using freshly grown bacteria in 4–5 ml sterile water and the turbidity was adjusted to 0.5 McFarland standards. This adjusted inoculum was spread over the entire surface of the Mueller-Hinton agar using a sterile swab stick to produce uniform growth[15]. The antibiotic susceptibility test was carried out by placing antibiotic disks aseptically namely Ceftazidime (CAZ 10), Doripenem (DOR 10), Cefixime (CFM 10), Cefuroxime (CXM 30), Cefoxitin (CX 30), Cefazolin (CZ 30), Ceftriaxone (CTR 10), Moripenem (MRP 10), Ampicillin (A/S 10), Ertapenem (ETP 10), Amoxyclav (AMC 30) and Aztreonem (AT 30) with the help of sterile forceps on the grown Mueller-Hinton agar incubating for 24 h at 37 ◦C.

The zone of inhibition at 37 ◦C was measured with help of milliliter ruler and the results were documented according to Clinical Laboratory Standards Institute and considered as sensitive, intermediate and resistant. Bacteria that are resistant to two or more different classes of antibiotics, are multidrug-resistant.

Extraction of genomic DNA for molecular identification: Four bacterial isolates out of forty-six phenotypically examined isolates were selected based on their antibiotic susceptibility pattern for isolation of bacterial Genomic DNA with 24 hr old grown cultures in nutrient broth. The isolation protocol used for isolation includes following steps: The sample was picked up and mixed with 1 ml of extraction buffer. Homogenate was transferred to microfuge tube. Add equal volume of phenol: chloroform:

isoamlyalcohol in the ratio 25:24:1 to the tubes and mix well. Then centrifuge the tubes at room temperature for approximately 15 min at 14,000 rpm. Collect upper aqueous phase in a new tube and add equal volume of chloroform: isoamyl alcohol (24:1) and mix properly. Centrifuge the tubes at 10,000rpm for 10mints at room temperature and transfer the aqueous phase in new MCT.

Precipitate DNA by adding 0.1 volume of sodium acetate in 3M concentration at pH 7.0 with 0.7 volume of isopropanol. Incubate micro centrifuge tubes for 15 minutes at room temperature. After incubation, centrifuge tubes at 14,000 rpm. Now collect the DNA pellet and wash it with absolute ethanol and air dry the pellet after centrifugation. Now, dissolve the pellet in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA, add 5 µl of DNAse free RNAse A (10 mg/ml)[8].

Polymerase chain reaction and gel electrophoresis: Amplification of isolated DNA was performed for selected four isolates out of forty six multi-drug resistant bacteria using PCR. 50 microliter of reaction mixture was prepared with DNA 1 ul, 16s forward and reverse primer both 2 ul each, dNTPs (2.5mM each) 4 ul, 10X Taq DNA polymerase assay buffer 10 ul, Taq DNA polymerase enzyme (3U/ ml) 1 ul and water 30 ul. Then the samples were assayed in Biorad T100 thermal cycler and allowed to run. The cycling steps of the PCR were: Initial Denaturation for 3 minutes at 94°C, denaturation for 1 minute at 94°C with 30 cycles, annealing for 1 minutes at 50°C, extension for 2 minutes at 72°C and final extension 7 minutes at 72°C, for 35 cycles.

All amplified products were loaded on 1.5% agarose gel at 75 volts for 30 min in Tris-borate ethylene diamine tetra acetic acid (TAE) buffer and then stained with intercalating dye ethidium bromide[16]. The primer used, its sequences, melting temperature and its GC-content are shown in table 1.

Sequencing and Phylogenetic Analysis: The PCR amplified products were purified and sequenced at Biokart India Pvt. Ltd. using applied biosystems bigdye version ABI 3130 Genetic Analyzer. The obtained fasta sequence were analyzed by the BLAST program on NCBI site (https://www.ncbi.nlm.nih.gov/). Four of the sequences were submitted in NCBI to get accession numbers. Alignment of the sequences was done with the help of MEGA X software, followed by BLAST (Basic Local Alignment Search Tool) to determine the homology of a DNA sequence with the already stored available data in Genbank NCBI (National Center for Biotechnology Information).

Phylogenetic tree was constructed using distance matrix method. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000[12].

Results and Discussion

In this study, 46 bacterial isolates were isolated from different sections of different hospitals. Table 2 shows the distribution and characterization of identified isolates according to Bergey's Manual of Systemic Bacteriology.

Bacterial diversity: In the present work, we observed that *Staphylococcus* and *Klebsiella* species have the highest occurrence among another species of bacteria. These are the two most prominent strains that we have found in hospitals responsible for causing nosocomial infections in hospital environment.

Antibiotic resistance pattern: Antibiotics are generally grouped into 2 groups based on their activity spectrum that is broad spectrum antibiotics and narrow spectrum antibiotics. Both groups of antibiotics play an important role in the treatment of patients where causative agent is not known.

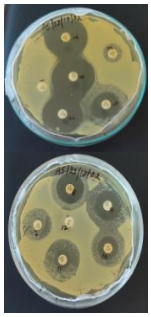
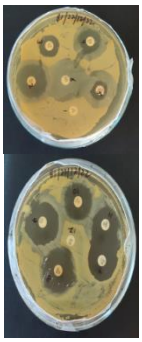
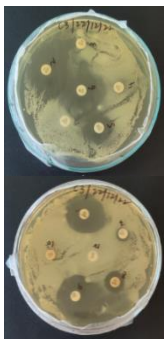
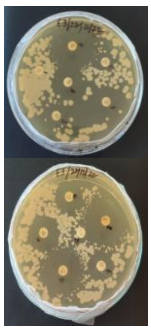
Table 1
Details of primers used

S.N.	Oligo Name	Sequence (5`à 3`)	Tm (°C)	GC- Content
1.	16s Forward	GGATGAGCCCGCGGCCTA	57	72.22%
2.	16s Reverse	CGGTGTGTACAAGGCCCGG	58	68.42%

Table 2
Identification and Distribution (%) of Bacterial Isolates

Organism	Maternity section	Surgical ward	Washroom area	Testing labs,	Common hall area	Total
Staphylococcus aureus	2	1	5	2	6	16(35%)
Proteus sp	0	0	2	4	2	8(17%)
Salmonella sp	0	0	0	1	0	1(2%)
Klebsiella sp	1	3	4	2	1	11(24%)
Enterobacter sp	0	1	1	1	0	3(7%)
Bacillus sp	0	1	3	1	2	7(15%)

Table 3
Sensitivity of different cultures with zone of inhibition

S.N.	Culture	Picture	Antibiotic Disk	Zone of Inhibition (mm)	Resistant/Sensitive
1.	A5		Ceftazidime (CAZ 10)	-	R
			Doripenem (DOR 10)	26mm	S/R
			Cefixime (CFM 10)	-	R
			Cefuroxime (CXM 30)	16mm	R
			Cefoxitin (CX 30)	25mm	S/R
			Cefazolin (CZ 30)	28mm	S
			Ceftriaxone (CTR 10)	-	R
			Moripenem (MRP 10)	19mm	S/R
			Ampicillin (A/S 10)	17mm	S/R
			Ertapenem (ETP 10)	12mm	R
			Amoxyclav (AMC 30)	10mm	R
			Aztreonem (AT 30)	-	R
2.	B1		Ceftazidime (CAZ 10)	-	R
			Doripenem (DOR 10)	21mm	S/R
			Cefixime (CFM 10)	11mm	S/R
			Cefuroxime (CXM 30)	14mm	R
			Cefoxitin (CX 30)	10mm	R
			Cefazolin (CZ 30)	-	R
			Ceftriaxone (CTR 10)	21mm	S/R
			Moripenem (MRP 10)	23mm	S
			Ampicillin (A/S 10)	22mm	S
			Ertapenem (ETP 10)	19mm	S/R
			Amoxyclav (AMC 30)	20mm	S/R
			Aztreonem (AT 30)	-	R
3.	C3		Ceftazidime (CAZ 10)	-	R
			Doripenem (DOR 10)	22mm	S/R
			Cefixime (CFM 10)	-	R
			Cefuroxime (CXM 30)	-	R
			Cefoxitin (CX 30)	14mm	R
			Cefazolin (CZ 30)	10mm	R
			Ceftriaxone (CTR 10)	-	R
			Moripenem (MRP 10)	11mm	R
			Ampicillin (A/S 10)	22mm	S
			Ertapenem (ETP 10)	-	R
			Amoxyclav (AMC 30)	23mm	S
			Aztreonem (AT 30)	-	R
4.	E3		Ceftazidime (CAZ 10)	-	R
			Doripenem (DOR 10)	26mm	S/R
			Cefixime (CFM 10)	11mm	R
			Cefuroxime (CXM 30)	19mm	S/R
			Cefoxitin (CX 30)	14mm	R
			Cefazolin (CZ 30)	22mm	S/R
			Ceftriaxone (CTR 10)	20mm	R
			Moripenem (MRP 10)	29mm	S
			Ampicillin (A/S 10)	16mm	S/R
			Ertapenem (ETP 10)	27mm	S
			Amoxyclav (AMC 30)	20mm	S/R
			Aztreonem (AT 30)	-	R

We have tested out isolated culture against beta lactam group of antibiotics namely Ceftazidime (CAZ 10), Doripenem (DOR 10), Cefixime (CFM 10), Cefuroxime (CXM 30), Cefoxitin (CX 30), Cefazolin (CZ 30), Ceftriaxone (CTR 10), Moripenem (MRP 10), Ampicillin (A/S 10), Ertapenem (ETP 10), Amoxyclav (AMC 30) and Aztreonem (AT 30) and we have found that 4 isolates were 100% resistant against two antibiotics i.e. ceftazidime and aztreonem and were further selected for molecular characterization[2].

Polymerase chain reaction and gel electrophoresis: The amplified bands of genomic isolated DNA for selected four isolates are given in fig. 1.

Phylogenetic Analysis and Evolutionary Relationships: After 16s rRNA sequencing analysis, it was observed that the three isolates namely A5, B1, E3 belongs to *staphylococii* group whereas C3 belong to bacilli group. Isolate A5 was found to be *Staphylococcus gallinarum* strain BTA5 16S ribosomal RNA gene with sequence ID: OQ978222.1 which was closest homologue to *Staphylococcus gallinarum* strain AB328 16S ribosomal RNA gene.

Isolate B1 was found to be *Mammaliicoccus sciuri* strain ADMS5 16S ribosomal RNA gene with sequence ID: OQ919246.1 and closest homologue to *Staphylococcus sciuri* strain TY-42 16S ribosomal RNA gene. Isolate E3 was also *Staphylococcus* sp. strain MKY 16S ribosomal RNA gene having sequence ID: MH100684.1 and the next closest homologue was found to be *Staphylococcus sciuri* strain TY-42 16S ribosomal RNA gene whereas isolate C3 was found to be *Bacillus firmus* strain T20 16S ribosomal RNA gene with sequence ID: MT457466.1 whose closest homologue was found to be *Bacillus oceanisediminis* strain C26 16S ribosomal RNA gene (Fig. 2a, b, c and d)) respectively[10].

The sequences obtained after Sanger sequencing were submitted to genbank of NCBI and accession numbers for our bacterial isolates A5, B1, C3 and E5 were provided as PP087998, PP087999, PP088000 and PP088001 respectively.

Conclusion

Studying MDR bacteria is getting importance day by day because of their antibiotic resistance increasing among different species of bacteria. Genes of MDR are transferring among different species of bacteria which ultimately lead to increased genetic diversity and biological diversity. Due to their high microbial diversity, culturing of these microbes is getting difficult because they are most abundant in the environment which significantly cause hurdle in microbial research.

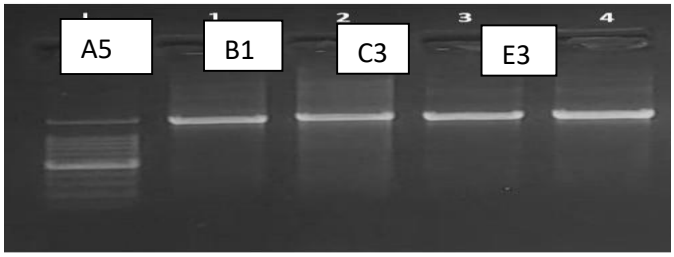


Fig. 1: PCR image of amplified DNA

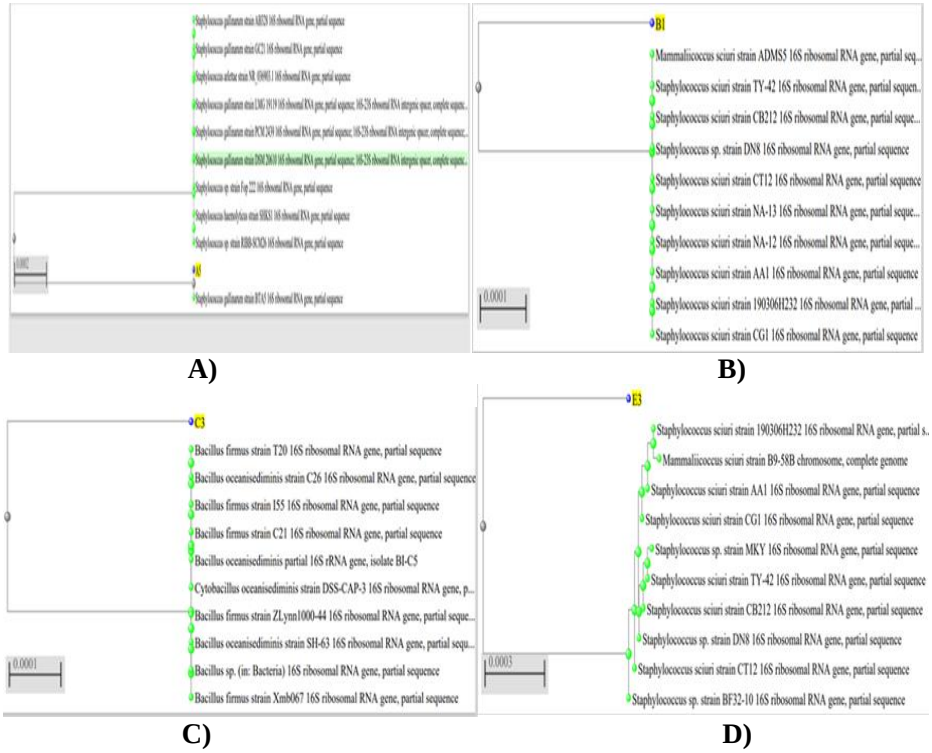


Figure 2: (A, B, C and D) Representing the phylogenetic tree of isolated microbes A5, B1, C3 and E3 by using distance-matrix method.

Maintenance of bacterial step is a very crucial step for developing any application of different microbes. So, the results of present study, indicate that bacterial culture combined with 16S rDNA sequence analysis provides a novel means of comparing multiple bacterial community structures present in MDRs[7]. The best way to manage nosocomial infections in hospital environment is to control colonization or infection with MDR bacteria. Although it is not ideal to carry out complete prevention of MDROs in the community is an unreasonable goal. For this, we need many multi-faceted approaches for the management and treatment of these MDROs but if they get identified, they need to be properly monitored. Infection can be controlled among staff as well as in the hospital environment. There is a need of appropriate antimicrobial therapy in order to offend pathogens antimicrobials susceptibility. Unlikely, the impact and burden of these MDROs especially belonging to *Staphylococcii* species in the hospital settings are increasing day by day.

There is a need of developing novel and safe antimicrobials therapies which has increased activity against MDROs. New methods and evidence based infection control practices by multi drug resistant bacteria could be rapidly identified before patients get admitted or enter in hospital settings in order to prevent influence of increasing nosocomial infections.

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