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ISOLATION, CHARACTERIZATION & BIOCHEMICAL ANALYSIS OF MULTIDRUG-RESISTANT BACTERIA FROM SOIL SAMPLES COLLECTED FROM HOSPITAL WASTE AND THEIR MOLECULAR CHARACTERIZATION USING 16S RDNA

Iqbal Hussain Mir*[1], Puja Baba[1], Pratibha Singh[1], Dr. Mukesh Sharma[1] and Ajeet Singh[2]

[1]Department of Biotechnology, Maharaj Vinayak Global University, Jaipur, Rajasthan, India.
[2]Helix Bio Genesis Pvt. Ltd., Noida, Utter Pradesh, India.

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***Corresponding Author**
Iqbal Hussain Mir
Department of Biotechnology, Maharaj Vinayak Global University, Jaipur, Rajasthan, India.

ABSTRACT

The antibiotic sensitivity test was performed for the isolated cultures obtained from different hospital waste of Delhi & NCR. During the study, out of 25 cultures, only 5 samples showed a remarkable antibiotic resistance. The bacterial isolates were characterized biochemically with the help of Bergey's manual. They included; Neisseria mucosa, Staphylococcus epidermidis and *Morecoccus cerebrosus. The bacterial isolates were further confirmed by molecular characterization using 16s rDNA. The total of 11 antibiotics were used included; Tetracycline, Ampicillin, Sparfloxacin, Co-timoxazole, Gatifloxacin, Cefrizoxime, Amoxicillin, Ciprofloxacin, Nitrofurantoin, Ofloxacin and Streptomycin. All strains were found resistant to Tetracycline, Ampicillin Sparfloxacin, Co-timoxazole and Gatifloxacin. 8 antibiotics showed an intermediate resistance. All isolates were found sensitive to Nitrofurantoin, Ofloxacin and Streptomycin.*

KEYWORDS: MDRO's, Hospital waste, Antibiotics, 16s rDNA.

INTRODUCTION

Microorganisms are everywhere a largely unseen world of activities that helped to create the biosphere and that continue to support the life processes on earth. Microorganisms that are resistant to one or more therapeutic classes of antimicrobial agents are called **Multidrug-resistant organisms (MDROs)**. Multiple drug resistance or Multidrug resistance is a

condition enabling a disease-causing organism to resist distinct drugs or chemicals of a wide variety of structure and function targeted at eradicating the organisms. Pathologic cells, including bacterial and neoplastic cells can display multidrug resistance or multiple drug resistance. Antibiotic resistance occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infectious. The bacteria survive and continue to multiply causing more harm. Bacteria can do this through several mechanisms. Some bacteria develop the ability to neutralize the antibiotic before it can do harm, others can rapidly pump the antibiotic out, and still others can change the antibiotic attack site so it cannot affect the function of the bacteria.

The number of MDROs will increase if the selective pressure of antibiotic use continues and the resistant organisms are able to spread from one person to another. Now, multiple-drug resistant (MDR) bacteria are a big public health problem in our world. It is really urgent to improve the current strategies to control this global public health threat.

Several studies have evaluated the microbiological content of hospital and household waste quantitatively and qualitatively and found that general hospital waste contains microorganisms with pathogenic potentials for humans comparable to household waste.[1] Most of the microorganisms have also been reported to be resistant to the commonly used antibiotics and as such have led to the outbreak of several diseases/infections.[2] Hospital effluent could contain multidrug resistant (MDR) enterobacteria and enteric pathogens which could pose a grave problem for communities. The antimicrobial selective pressure through indiscriminate use of antibiotics has played a significant role in enriching the MDR strains in the hospital practice. Present time the antibiotic resistance has become a major problem in the clinical and public health prospects.[3] The main risk for public health is that resistant genes are transferred from environmental bacteria to human pathogens. Waste effluent from hospitals contains high numbers of resistance bacteria and antibiotic residues at concentration able to inhibit the growth of susceptible bacteria.[4] Although sewage treatment processes reduce the numbers of bacteria in wastewater, the effluent will still generally contain large numbers of both resistant and susceptible bacteria.[5]

The present study is an attempt to isolate and characterize the multi-drug resistant (MDR) bacteria from hospital waste which was obtained from various hospitals of Delhi & NCR. The isolates were investigated for their sensitivity against eleven antibiotics and to check the activity of culture in the presence of various antibiotics.

MATERIAL AND METHODS

Sample Collection

The soil samples were taken from hospital wastage dumping sites. Soil sample was chosen because of higher probability of finding bacterial strains of localized zone mainly obtained from dump hospital wastage which may include medicines, edibles, patient's dressings etc, so there might be probability of finding large amount of pathogenic bacteria. Nearly, twenty five different soil samples were collected from various hospitals of Delhi & NCR. The bacteriological analysis of these samples was done by serial dilution and agar plate culture techniques. The obtained pure cultures were characterized based on their morphological and biochemical characteristics as described in Bergey's Manual for bacteriology.[6]

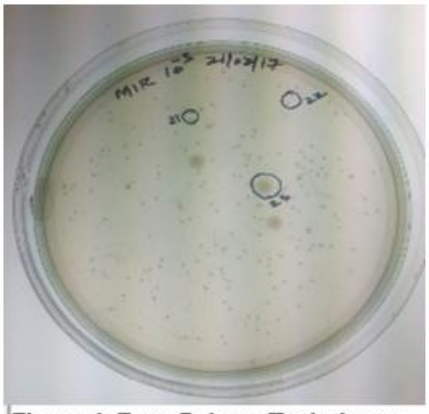

Figure 1: Pure Culture Techniques For Samples C21, C22 & C23.

Serial Dilution and Agar Plating Technique

This method is based on the principle that when soil sample containing bacterial colonies are cultured, bacterium develops into a visible colony on the nutrient agar plate. One gram of the collected soil sample was suspended in 9ml of saline to obtain a 10^{-1} dilution (10 times dilution). From the above dilution 1ml was transferred to a fresh 9ml saline solution to obtain a 10^{-2} dilution. The process was repeated in order to produce 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} serial dilutions. From the dilutions ranging from 10^{-3} to 10^{-6} 0.1 ml of the suspensions were added to nutrient agar plates (each dilution in 3 replicates) under sterile conditions and incubated at 37°C for 24 hours. The number of bacteria in the testes soil sample can be calculated by the following formula:

Organisms per millilitre per gram soil = number of colonies (average of 3 replicates)/ volume plated (0.1) x dilution.

The isolated cultures were differentiated by their morphological characteristics and transferred to fresh nutrient agar media to produce in pure form.

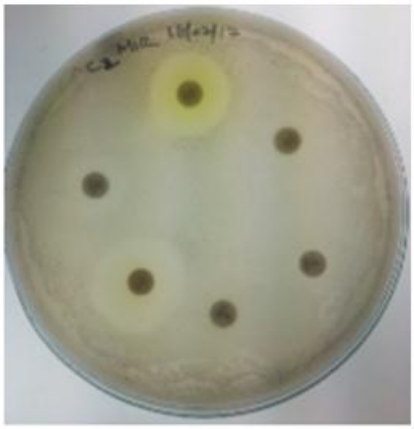

Figure 2: Antibiotics sensitivity test for C2 culture

Antimicrobial resistance & susceptibility profiling of organisms using different antibiotics

The standard method of Kirby-Bauer by disk diffusion was employed to investigate the antibiotic susceptibility profiles of the bacterial isolates.[7] The antibiotic discs used in this experiment are given in the following (Table 1). The Muller Hinton agar was allowed to solidify in the Petri plates for the purpose of our experiment. Sample 1, 2 and 3 previously inoculated into nutrient broths were spread evenly on the MH plates. Antibiotics discs of the above mentioned concentration were placed carefully on the plates and left for diffusion for some time. The plates were then incubated for 24 hours at 37°C.

The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation at 37°C, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk. The zone around an antibiotic disk that has no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate. Clear zones of growth inhibition were measured in millimetres by a ruler.

Table 1: Commercial antibiotics (HiMedia Laboratories Pvt. Ltd.) used for susceptibility of bacterial isolates.

S. No.	Antibiotics	Antibiotic Code	Antibiotic Disc Concentration	Antibiotic Class	Antibiotic Action
1	Ampicillin	AM	10 mcg	Penicillin	Cidal*
2	Amoxicillin	AX	25 mcg	Penicillin	Cidal
3	Ciprofloxacin	CIP	5 mcg	Fluorquinolones	Cidal
4	Ofloxacin	OF	5 mcg	Fluorquinolones	Cidal
5	Sparfloxacin	SPX	5 mcg	Fluorquinolones	Cidal
6	Gatifloxacin	GAT	5 mcg	Fluorquinolones	Cidal
7	Streptomycin	STR	10 mcg	Aminoglycosides	Cidal
8	Cotrimoxazole	CoT	25 mcg	Sulfonamides	Cidal
9	Ceftrizoxime	CZX	30 mcg	Cephalosporins	Cidal
10	Nitroflurantoin	NIT	300 mcg	Nitrofurantoin	Static
11	Tetracyclin	TE	30 mcg	Tetracycline	Static

*Cidal= Bacteriocidal; Static = Bacteriostatic

Characterization of Bacterial Culture

Gram staining of bacteria

A smear of the microorganisms grown in NB was prepared on clean grease free glass slide by a sterile/flamed inoculating loop after cooling it. Smear was allowed to air dry and then heat fixed. Smear was flooded with crystal violet & allowed to stand for 1 minute and then gently washed with tap water. Smear was flooded with Gram Iodine and allowed to stand for 1 minute. Smear was decolorised / washed with 95% ethyl alcohol until alcohol runs almost clear. Smear was counterstained with Safranin for 1 minute. Gently washed with tap water, air dried & observed under compound microscope at 40X magnifications.

Biochemical characterization of the isolated bacterial colonies and growth in MacConkey agar

Total of twenty seven biochemical tests were performed according to Bergey's Manual for bacteriology to characterize the unknown bacterial cultures. Initially an unknown bacterial culture was checked for catalase and oxidase activity.[8] The reagent used in oxidase test was N, N-dimethyl-p-phenylenediamine (DMPD). The presence of catalase enzyme in the test isolate was detected using hydrogen peroxide. The catalase test was done by placing a drop of hydrogen peroxide on a microscope slide. Carbohydrate fermentation tests were used to detect the ability of microorganisms to ferment a specific carbohydrate. In our experiment, we used glucose, galactose, mannose, lactose, maltose glycerol, mannitol and sucrose with the multi drug resistant (MDR) organism isolated. Indole test was carried out by adding few

drops of Kovac's reagent in peptone water broth with bacterium incubated for 18-24 hours at 37°C. In Voges Proskauer (VP) test, 40% KOH and alpha-nephthol were added to test broth after incubation and exposed to atmospheric oxygen. Nitrate reduction test was done by adding 1ml of each sulphanilic acid and α-nepthylamine to the inoculated test organisms to detect the production of enzyme nitrate reductase which reduces nitrate to nitrites. Starch hydrolysis test was also carried out to check the presence of and exoenzyme amylase which cleaves the starch into disaccharides and monosaccharides. This was done by flooding Iodine reagent to the overnight culture medium containing starch. Similarly, gelatinase test was carried out to check the presence of gelatinase.

With some knowledge about the nature of organisms we moved towards the task for growing the selected MDR on the following media. For this, MacConkey agar culture medium was used.[8] The bacterial colonies isolated were subjected to catalase and oxidase test and observations were noted. The MDR's obtained were made to grow in MacConkey colony and characteristics were noted down.

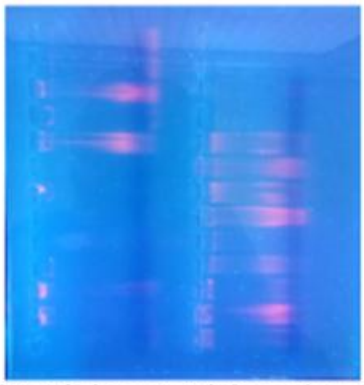

Figure 3: Agarose gel electrophorasis of DNA Samples of bacterial isolates C2, C3, C18, C22 and C24.

Extraction of DNA and Molecular Characterization Using 16s rDNA

Bacterial genomic DNA was isolated as per the standard protocol.[9] The isolated cultures were streaked on Luria Betani (LB) agar and grown overnight. Cells were harvested from 5 mL of the culture and to this 100µL of lysozyme was added and incubated at RT for 30 min, followed by the addition of 700 µL of cell lysis buffer (Guanidium isothiocyanate, SDS, TrisEDTA). The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent. 700 µL of isopropanol was added on top of the solution. The two layers were mixed gently till white strands of DNA were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in

50µL of 1X TE buffer. The quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide (0.5 µg/µL). A single intense band with slight smearing was noted. The extracted genomic DNA was used as template DNA for amplification of the 16S rDNA gene. PCR amplification of 16S rRNA gene: PCR reaction was performed in a gradient thermal cycler (Thermo Scientific). The reaction mixture of 50 µl consisted of 10 ng of genomic DNA, 3 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM TrisHCl, 500 mM KCl pH-8.3), 200µM dNTP, 10 p moles each of the two universal primers and 1.5mM MgCl2. The 16S rDNA sequence was amplified using universal primers (Forward primer 5'- AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5 (5’ GGTTACCTTGTTACGACTT 3’). The reaction condition include 1 min denaturation (95°C) followed by 30 cycles of 96°C for 30 s, 48.5°C for 30 s and 72°C for 30 s and a final extension of 72°C for 10 min. PCR products were then separated and visualized on 1% agarose gel electrophoresis to confirm amplification.

Samples thus were sent for DNA sequencing to determine the precise sequence of amplified product. The amplified product was sequence using the same universal primers used for amplification. The sequencing was done using ABI 3500 Genetic Analyser^{TM} with Big Dye Terminator version 3.1. Applied Biosystems Micro Amp Optical 96-well reaction plate was used for the sequencing. Bioinfomatic analysis of sequence data (FASTA format) was done for genebank search and sequence alignment assay. Nucleotide BLAST was performed for this purpose and E-Value, % similarity and local alignment were taken into consideration for determining the homology and to identify the bacterial strains upto species level.

RESULTS AND DISCUSSION

Twenty five bacterial isolates were selected for antibiotic resistance, morphological, biochemical and further investigations. These bacterial isolates were screened for antimicrobial sensitivity profiles. Out of the twenty five different samples only five samples showed a remarkable antibiotic resistance and these five samples were labelled as C2, C3, C18, C22 and C24. Resistance range of the bacterial isolates for 11 antibiotics examined in downward order was separately tetracyclin, Ampicillin, Sparfloxacin, Co-timoxazole, Gatifloxacin, Cefrizoxime, Amoxicillin, Ciprofloxacin, Nitrofurantoin, Ofloxacin and Streptomycin. The resistance of the 5 bacterial isolates to the 11 commonly used antibiotics revealed that for all antibiotics, all the cultures were showing resistance against Tetracycline, Ampicillin Sparfloxacin, Co-timoxazole and Gatifloxacin. Moreover, most isolates showed

intermediate resistant to 8 antibiotics out of total 11 investigated. All isolates were found sensitive to Nitrofurantoin, Ofloxacin and Streptomycin. Antibiotic susceptibility pattern of the bacterial isolates is summarized below in Table 2.

Five MDR bacterial cultures were isolated from soil samples collected from hospital waste of Delhi & NCR which were selected for further characterization. Based upon Gram staining, three isolates (C2, C18 and C24) were identified as Gram negative which are cocci and two isolates (C3 and C22) were Gram positive which are cocci also. All isolated bacterial cultures were aerobic. Based upon the biochemical analysis (Table, 3), bacterial isolate labbled as C2 was identified as *Neisseria mucosa*, bacterial isolates labbled as C3 and C22 was identified as *Staphylococcus epidermidis* and bacterial isolates labbled as C18 and C24 was identified as *Morecoccus cerebrosus.* The use of 16S rDNA gene sequences to identify new strains of bacteria is gaining momentum in recent years. Based upon the molecular characterization while using 16S rDNA gene sequence to characterize the bacterial isolate, the homology search made using BLAST showed 97% identity with that of *Neisseria mucosa* strain labelled as C2, NCBI Gene Bank Accession No: CP028150.1 and E-value equal to 0 for all closely related taxa, 98% maximum similarity with *Staphylococcus epidermidis* strains labelled as C3 and C22, NCBI Gene Bank Accession No: CP014119.1 and E-value equal to 0 for all closely related texa and 98% similarity with *Morecoccus cerebrosus* strains labelled as C18 and C24, NCBI Gene Bank Accession No: LN899799.1 and E-value equal to 0 for all closely related texa.

Table 2: Antibiotic susceptibility test of 5 bacterial cultures.

Antibiotic discs	C2	C3	C18	C22	C24
Tetracyclin	R*	R	R	R	R
Ampicillin	R	R	R	R	R
Sparfloxacin	R	R	R	R	R
Cotrimoxazole	R	R	R	R	R
Gatifloxacin	R	R	R	R	R
Cefrizoxime	R	R	R	S	R
Amoxicillin	R	R	R	S	S
Ciprofloxacin	R	S	S	R	S
Nitroflurantoin	S	S	S	S	S
Ofloxacin	S	S	S	S	S
Streptomycin	S	S	S	S	S

*R = resistant; S = sensitive

Table 3: Characteristic tests of multidrug-resistant bacterial isolates.

S.NO.	Test	C2	C3	C18	C22	C24
1	Gram's Staining	-ive, Cocci	+ive, Cocci	-ive, Cocci	+ive, Cocci	-ive, Cocci
2	Diameter > 5mm	-ive*	-ive	-ive	-ive	-ive
3	Caretenoid Pigment Production	-ive	-ive	-ive	-ive	-ive
4	Aerobic Growth	+ive	+ive	+ive	+ive	+ive
5	Anaerobic Growth	+ive	+ive	+ive	+ive	+ive
6	Growth at 15°C	-ive	-ive	-ive	-ive	-ive
7	Growth at 45°C	+ive	+ive	+ive	+ive	+ive
8	Growth @10% NaCl	-ive	-ive	-ive	-ive	-ive
9	Growth @ 15% NaCl	-ive	-ive	-ive	-ive	-ive
10	Nitrate Reduction	-ive	+ive	+ive	+ive	+ive
11	Acetoin Production (V.P.)	+ive	+ive	+ive	+ive	+ive
12	Urease Hydrolysis	-ive	+ive	-ive	+ive	-ive
13	Esculine Hydrolysis	+ive	+ive	-ive	+ive	-ive
14	Catalase	+ive	+ive	+ive	+ive	+ive
15	Oxidase	+ive	-ive	+ive	-ive	+ive
16	Novobiocin resistance (1.6 µg)	+ive (sen)	-ive (resi)	+ive (sen)	-ive (sen)	+ive (sen)
17	Acid Production From					
(a)	Fructose	+ive	+ive	+ive	+ive	+ive
(b)	Galactose	+ive	+ive	-ive	+ive	-ive
(c)	Glucose	+ive	+ive	+ive	+ive	+ive
(d)	Lactose	-ive	+ive	-ive	+ive	-ive
(e)	Maltose	+ive	+ive	+ive	+ive	+ive
(f)	Glycerol	+ive	+ive	-ive	+ive	-ive
(g)	Mannitol	+ive	-ive	-ive	-ive	-ive
(h)	Sucrose	+ive	+ive	+ive	+ive	+ive
(i)	Mannose	-ive	+ive	-ive	+ive	-ive
18	Motality	Non Motile	Non Motile	Non Motile	Non Motile	Non Motile
19	Fluorescent Pigment	-ive	-ive	-ive	-ive	-ive
20	Non Fluorescent diffusion Pigment	-ive	-ive	+ive	-ive	+ive
21	Growth at 4°C	-ive	-ive	-ive	-ive	-ive
22	Growth on MacConkey Agar	+ive	+ive	+ive	+ive	+ive
23	H_2S Production	-ive	-ive	+ive	-ive	+ive
24	Indole Production	-ive	-ive	-ive	-ive	-ive
25	Starch Hydrolysis	-ive	-ive	-ive	-ive	-ive
26	Gelatin Hydrolysis	-ive	-ive	-ive	-ive	-ive
27	Haemolysis	-ive	-ive	+ive	-ive	+ive
	Identified Isolates	***Neisseria mucosa***	***Staphylococcus epidermidis***	***Morecoccus cerebrosus***	***Staphylococcus epidermidis***	***Morecoccus cerebrosus***

*+ive = presence; -ive = absence

CONCLUSION

A major factor in the emergence of Multi Drug Resistant Organisms (MDRO's) is overuse of antibiotics in any setting, the hospital or the community. Many human pathogenic bacteria are examples of this emerging crisis, and they are extremely difficult to treat with present existing extended-spectrum antibiotics, leading to increased morbidity and mortality in human. The population and pollution of the whole world is increasing day by

day. The main source of the Multi Drug Resistant Organisms is the hospital wastes. In the metro cities like New Delhi, the hospitals and the clinics are found in every knock and corner and are increasing. Thus Multi Drug Resistant Organisms are increasing in such cities and is becoming a serious threat in the whole world. It is really urgent to improve the current strategies to control this global public health threat.

In our present study we have tried to isolate and identify using both biochemical and molecular methods to ascertain the MDR microbes upto species level. We have also done the detailed analysis of MDR to understand and decide the possible control methods for the above mentioned strains/microbes.

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