Isolation of MDR Pathogens Isolated From Hospital Area's: Evaluate Their Activty Against Some Herbal Plants

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Abstract: Antibiotic resistant bacteria was isolated from General Hospital near Rewa District M.P.). The microscopical, biochemical tests suggested it to be *Neisseria sp.* Our major aim was to check the effect of several aqueous plant extracts on MDR and finding out tentative MIC value, study of any physiological changes brought about by the natural extract and ultimately to study activity executed by the most successful plant extract on the multidrug resistant bacteria. A total of three isolated were identified and purified from the samples, further screened for individual antibiotics at their respective varying concentrations and all the three isolates were found to be strong resistant against antibiotics selected in the study. Morphological, biochemical and physiological properties were analysed for all the isolates.

Keywords: MDR pathogens, hospital samples, *Neisseria sp*, drug resistance.

1.Introduction:

Since the 1940s, these drugs have greatly reduced illness and death from infectious diseases. Antibiotic use has been beneficial and, when prescribed and taken correctly, their value in patient care is enormous. However, these drugs have been used so widely and for so long that the infectious organisms the antibiotics are designed to kill have adapted to them, making the drugs less effective. Many fungi, viruses, and parasites have done the same. Some microorganisms may develop resistance to a single antimicrobial agent (or related class of agent), while others develop resistance to several antimicrobial agents or classes. These organisms are often referred to as multidrug-resistant or MDR strains. In some cases, the microorganisms have become so resistant that no available antibiotics are effective against them [1]. Large amounts of antibiotics used for human therapy resulted in the selection of pathogenic bacteria resistant to multiple drugs. Multidrug resistance in bacteria may be generated by one of two mechanisms

An antibiotic is a drug used to treat infections caused by bacteria and other microorganisms. Originally, antibiotic was defined as a substance produced by one microorganism that selectively inhibited the growth of another. Synthetic antibiotics, usually chemically related to natural antibiotics, have since been produced that accomplish comparable tasks. There are several major classes of antibiotics that can be categorized based on their mode of antibacterial action. In general, antibiotics can be categorized as those that inhibit cell wall synthesis, protein synthesis, and nucleic acid synthesis [2-4].

'Selective pressure' refers to the environmental conditions that allow organisms with novel mutations or newly acquired characteristics to survive and proliferate. Mutations that increase an organism's resistance to antimicrobial agents occur naturally in bacteria. Exposure to a stimulus that inhibits or kills the susceptible majority of a bacterial population allows a resistant subset of strains to grow at the expense of susceptible organisms. A minority of strains present in a given setting may be resistant to the antibiotic being used. The selective factor is the antibiotic (usually) to which the sub-population is resistant. Hence, the phenomenon of antibiotic resistance is based on selection for organisms that have enhanced ability to survive doses of antibiotics that would have previously been lethal [5].

2. Materials and methodology:

2.1. Soil study, isolation, characterization of microorganism from hospital dumping site

The entire soil sample was well characterized and taken .The organisms obtained were first isolated in mixed culture and followed by pure culture techniques to use the pure cultures for future use. The Gram staining method were adopted for characterization in which out of the three selected microorganisms to get a better idea about the organisms and probable antagonistic role were checked by the bacteria producing prominent red pigment in mixed culture but not in pure culture probably due to lack of competition[6].

2.2.Isolation of microorganisms by Pour Plate culturing Method

100 µl of sample was poured onto the previously sterilized Nutrient Agar surface by a sterile spreader under aseptic condition. The plates were then placed in an incubator for 24 hours at 37ºC.

2.3.Pure Culture Maintenance by Streak Plate culture technique

The inoculating loop was sterilized by putting it in flame till red hot, after cooling it down, it was dipped into 95% ethyl alcohol and further heated for proper sterilization. Then taken three well distinguished colonies from spread plate were further streaked over three different Nutrient Agar plate surface by sterilized inoculating loop. These were the incubated for 24 hours at 37ºC [6-7].

3.Determination of isolated microorganism by Gram staining method

3.1.Procedure

A smear of the three different 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 decolorized / 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[7].

3.2.Antibiotic Sensitivity Test of micro-organisms using antibiotics and UV treatment:

3.2.1.Concentration of antibiotic discs

Antibiotic discs of ampicillin (10 mcg), tetracycline (30 mcg), Co-triamoxazole (25 mcg), nitrofurantoin (300 mcg) , streptomycin(10 mcg).

The Muller Hinton agar was allowed to solidify in the Petri plates for the purpose of our experiment. Colony 1, 2 and 3 previously inoculated into nutrient broths were spread evenly on the MH plates. Antibiotics of the above mentioned concentration were placed carefully on the plates and left for diffusion for some time. After that the plates were incubated at 37ºC and later observations were noted down[8].

3.2.2.UV irradiation

The organism found resistant (Colony 2) to the action of some antibiotics was subject to mid UV of 280-350 nm for 5 minutes in a short UV chamber [3]. The above antibiotic disc diffusion technique was carried out again and observations were noted.

4. Biochemical characterization of the isolated bacterial colonies :

Initially an unknown bacterial culture was checked for catalase and oxidase activity [4]. The reagent used in oxidase test was N, N-dimethyl-p-phenylenediamine (DMPD). The presence of catalase enzyme in the test isolate is detected using hydrogen peroxide. The catalase test was done by placing a drop of hydrogen peroxide on a microscope slide. Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific carbohydrate. In our experiment, we used glucose and sucrose with the Multi Drug Resistant (MDR) organism isolated. With some knowledge about the nature of organisms we moved towards the task for growing the selected MDR on the following media. Tryptic soy agar, Chocolate agar (CHOC) or Chocolate Blood Agar (CBA) and Mac-Conkey agar culture medium were used [4].

The bacterial colonies isolated were subjected to catalase and oxidase test and observations were noted. The MDR obtained was used to carry out carbohydrate fermentation test by using 6% glucose and 6% sucrose in nutrient agar. The MDR obtained was made to grow in Tryptic soy agar, Mac-Conkey and chocolate agar and colony characteristics were noted down.

4.1. Inhibition of multiple drug-resistant bacteria by different natural plant extracts

Due to the increase of resistance to antibiotics, there is a need to develop new and effective antimicrobial agents. Among the potential sources of new agents, plants have long been investigated because they contain many bioactive compounds that can be of interest in therapeutics. So, to combat the MDRs, different types of natural extracts are now used. In our experiment, tea, turmeric, tobacco, garlic, ginger, neem, tulsi, pepper, chilli were used to check whether the MDR growth is inhibited or not.

Plant extracts were made by grinding them in mortar pestle, both in water and alcohol at a specific concentration given as follows: Turmeric (0.25g/ml), Neem (2g/ml), Garlic and Ginger (0.5g/ml), Black pepper (0.2g/ml),Chilli(1g/ml), Tulsi (2g/ml), Clove(3g/15ml), Kalmegh(0.5g/ml) and Tobacoo(2.5g/10ml).Then with the help of funnel and filter paper, the crude extracts were filtered and taken in test tubes and beakers. The culture (Multiple drug resistant) was spread over the agar in petriplate and 3, 4 wells were made by borer. The natural extracts were then given into the well by micropipette each 200 µl. This experiment was also done by disc-diffusion method for some of the natural extracts. The plates were then kept overnight in incubator at 37ºC[9].

4.2.Determination of minimum inhibitory concentration (MIC) of garlic by cup- plate method

MIC scores are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. We checked for minimum inhibitory concentration of the aqueous extract of garlic bulbs on the isolated MDR to recommend an alternative way to control the pathogen.

4.3.Dilution of the Garlic extract:

Initially the stock concentration-1gm/ml of garlic was made and it was further diluted into different concentration according to the following table:

s.no	Concentration of garlic extract (g/ml)	Volume of sterile water added (ml)	Volume of garlic extract added from stock conc. (ml)
1.	0.1	1.35	0.15
2.	0.2	1.20	0.30
3.	0.25	1.125	0.375
4.	0.5	0.75	0.75

5.	0.75	0.375	1.125

100µl log phase culture (OD600=0.6) of the MDR organism was spread onto the MHA plate. Small cups were made with the help of puncher on those MHA plate and the cups were properly labeled.200µl of each concentration of garlic extract were poured in each cup of MHA plate. The plates were incubated in BOD shaker at 37oC for overnight.

5.Results:

5.1.Isolation of microorganisms by pure culture technique :

Many well characterized colonies are obtained but 3 different colonies are being considered for further analysis. We at the end chose only colony number two which showed antibiotic resistance against the five antibiotics.

5.1.1.Determination of isolated microorganism by Gram staining method

Figure 1: Gram negative cocci

The colony 2 shows no susceptibility towards the antibiotics

Colony	**Ampicillin (10 mcg)**	**Tetracycline (30 mcg)**	**Co-Triamoxazole (25 mcg)**	**Nitrofurantoin** (300 mcg)	**Streptomycin (10 mcg)**
2	No zone	No distinct zone	1.53 mm	No distinct zone	No zone

Table 1: Zone of inhibition given by the microorganism under 5 different antibiotics

Figure2: Presence of no zone of inhibition except Co-triamoxazole at the centre

5.2.Antibiotic diffusion test after UV exposure:

Under UV exposure the susceptibility of the microbe towards the antibiotics changed.

Colony	**Ampicillin**	**Tetracycline**	**Co-triamoxazole**	**Nitrofurantoin**	**Streptomycin**
2	2.12	0.86	1.54	0.28	1.16

Table 2: After UV exposure at mid UV (280-350nm), the microbe becomes susceptible

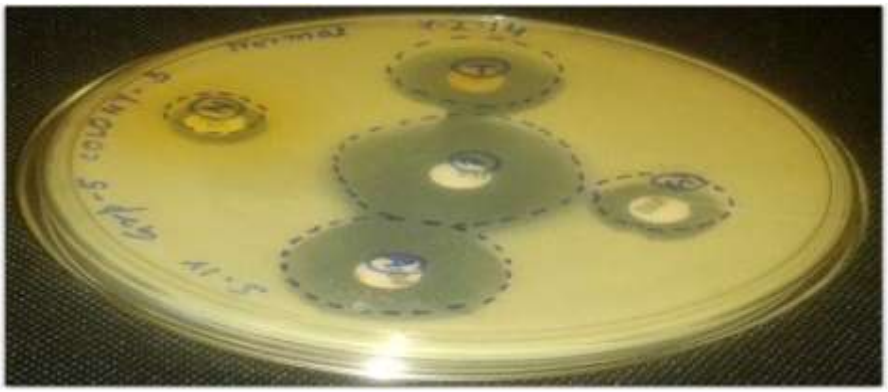

Figure 3: After UV exposure the susceptibility increases towards the antibiotics

5.3. Biochemical characterization of the isolated bacterial colonies :

Test	**Colony 2**
Catalase	+
Oxidase	+

5.4.Pattern of growth on selected media:

Type of media	**Morphology**	**Appearance**	**Remarks**
Tryptic Soy Agar	Small, opaque, raised. glistening and smooth	Colorless	Organism can be non-fastidious in nature
Mac-Conkey	Raised, small colonies	Pink	utilize lactose
Chocolate Agar	Raised, small colonies,having β-hemolytic colonies	Grayish-white	utilize lysed red blood cells

Table 3: Nature of growth in TSA, utilization of lactose (growth in Mac-Conkey and nature of growth in chocolate agar

Figure 4: Left petriplate showing the growth on TSA and right showing the growth on chocolate and Mac-Conkey agar

5.5. Inhibition of multiple drug-resistant bacteria by different natural plant extracts

Only garlic showed significant zone of inhibition against the MDR organism

S.NO.	Garlic (ZOI)	Tea (ZOI)	Ginger (ZOI)	Tobacco (ZOI)	Neem (ZOI)
1.	2.6	0.6	0.7	0.9	0.6
2.	1.6	0.6	0.6	0.7	0.6
3.	1.6	0.5	0.6	0.8	0.4

Table 4: Garlic (*Allium sativum* showing maximum zone of inhibition compared to others)

6. Determination of minimum inhibitory concentration (MIC) of garlic by cup- plate method

After overnight incubation the zone of inhibition were observed and from the inhibition zone MIC value was determined which is tabulated below:

Concentration of garlic extract (g/ml)	**Inhibition zone obtained (mm)**
0.1	-
0.2	10
0.25	18
0.5	24
0.75	32

1.0	36

7. The effect of garlic extract on the isolated MDR under inverted phase contrast microscope.

Single, small, round shaped bacterial (cocci), healthy cells were observed under compound microscope in control (no garlic extract) having pendulum like motility. But just after addition of garlic extract the motility slowed down and ultimately stopped.

8. Conclusion:

Hospital including operation theatre, ICU and patient ward are potent platform for spreading of pathogens among individuals. The bacterial isolates purified from those hospital samples showed resistance against commonly used drugs. Based upon the macroscopically studies, biochemical tests, and the colony characteristic on Nutrient agar, Tryptic soy agar, Chocolate agar and Mac-Conkey agar of the colony 2 we assumed the presence of *Neisseria sp*. It has been found that several non-pathogenic as well pathogenic strains of Neisseria are resistant to many antibiotics which we used such as β-lactamase producing *Neisseria* sp, tetracycline,streptomycin. It has also being reported that upon UV exposure the plasmid undergoes changes owing to mutations that make the MDR strains more susceptible towards the antibiotic. That is what we observed when we carried out the experiment with antibiotic discs after UV exposure. One way is to shift towards natural remedies which need a thorough screening of the local flora and ultimately going for the one which is easily accessible with minimal side effects and effective against the pathogen. The organism which was isolated from the hospital soil was resistant to many of the commonly used broad-spectrum antibiotics such as Streptomycin, tetracycline, nitrofurantoin, ampicillin. But this multiple-drug resistant organism is greatly inhibited by the garlic extract. The MIC score of garlic on the experimental MDR organism lie between 0.2-0.25g/ml. Even after the treatment with garlic extract, the motility of the MDR organism stopped. From this view point we may conclude that the isolated microorganism from hospital disposal site was sensitive to garlic extract and garlic in the form of many formulations can be taken to combat the growth of the MDR bacteria.

9.Reference:

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