Isolation and Characterization of Multi drug Resistant Super Pathogens from soil Samples Collected from Hospitals

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Abstract- Soil samples from two different city hospitals were collected pre-treated along with several antibiotics for primary screening of numerous microbes were cultured after serial dilution over sterile nutrient agar plates. A total of three isolated were identified and purified from the samples, further screened for individual antibiotics at their respective varying concentrations and found that isolates 2nd is strong resistant against antibiotics selected in the study. Morphological, biochemical and physiological properties were analysed for all the isolates.

Index Terms- MDR pathogens, Hospital samples, Antibiotic Drug resistance.

I. Introduction

A multi-drug resistant organism (MDRO) is a bacteria that is resistant to many antibiotics. If bacteria are "resistant" to an antibiotic it means that certain drug treatments will not work. It is important to prevent the spread of an MDRO. Infections caused by MDROs can be more difficult to treat, since there are fewer antibiotics that work against them.

Antibiotic-resistant pathogens constitute an important and growing threat to the public health. More than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the drugs most commonly used to treat these infections. In the past decade, there has been an increasing focus on the costs of medical care and it also has become clear that prolonged hospital stay and higher costs result from infections caused by antibiotic-resistant pathogens as compared with infections due to antibiotic-susceptible strains of the same species.

The World Health Organization (WHO) estimates that 32% of the world population is infected with Mycobacterium tuberculosis, the causative agent of tuberculosis, with 9.2 million new TB cases and 1.7 million deaths from TB reported in 2007. Drug resistance to isoniazid and rifampicin (the definition for multidrug-resistant (MDR) TB), the two most potent first-line antimicrobial drugs for the treatment of TB, is a persisting global problem with surveillance data indicating increasing trends in several countries. In 2007, the WHO reported the highest rates of MDR TB ever recorded, with up to 22% of new TB cases being resistant to both isoniazid and rifampicin in some areas of the former Soviet Union.

Despite the existence of effective drug treatments, tuberculosis (TB) causes 2 million deaths annually worldwide. Effective treatment is complicated by multidrug-resistant TB (MDR TB) strains that respond only to second-line drugs. We projected the health benefits and cost effectiveness of using drug susceptibility testing and second-line drugs in a lower-middle income setting with high levels of MDR TB. Antimicrobial resistance is not new, but the number of resistant organisms, the geographic locations affected by drug resistance, and the breadth of resistance in single organisms are unprecedented and mounting. Diseases and disease agents that were once thought to be controlled by antibiotics are returning in new leagues resistant to these therapies[1,2]. Drug-resistant strains initially appeared in hospitals, where most antibiotics were being used. Sulfonamide-resistant *Streptoccoccus pyogenes* emerged in military hospitals in the 1930s. Penicillin resistant *Staphylococcus aureus* confronted London civilian hospitals very soon after the introduction of penicillin in the 1940. Similarly, *Mycobacterium tuberculosis* with resistance to streptomycin emerged in the community soon after the discovery of this antibiotic[3,4]. Resistance to multiple drugs was first detected among enteric bacteria-namely, *Escherichia coli*, *Shigella* and *Salmonella* in the late 1950s to early 1960s[5]. Such strains posed severe clinical problems and cost lives, particularly in developing countries[6]. Nevertheless, the resistance problem was perceived by some, most notably those in the industrialized world, as a curiosity of little health concern confined to gastrointestinal organisms in distant countries[7-9]. This attitude changed in the 1970s when *Haemophilus influenza* and *Neisseria gonorrhoeae*, organisms that cause respiratory and genitourinary disease, respectively, emerged with resistance to ampicillin and, in the case of *Haemophilus*, with resistance to chloramphenicol and tetracycline as well. Fuelled by increasing antimicrobial use, the frequency of resistance escalated in many different bacteria, especially in developing countries where antimicrobials were readily available without prescription. Poor sanitation conditions aided spread and small healthcare budgets prevented access to new effective but more expensive antibiotics. Individuals may succumb to MDR infections because all available drugs have failed, especially in the developing world. Notable global examples include hospital and community MDR strains of *Mycobacterium tuberculosis*, *Enterococcus faecium*,

Enterobacter cloacae, *Klebsiella pneumoniae*, *S. aureus*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (World Health Organization website). In developing countries, MDR enteric disease agents such as *Salmonella Enteritidis*, *Shigella flexneri* and *Vibrio cholera* threaten and circumvent public health measures[10, 11]. Overall, in the United States and the United Kingdom, 40-60% of nosocomial *S. aureus* strains are methicillin-resistant (MRSA) and usually MDR. More deaths are associated with MRSA than with methicillin-sensitive strains. A steadily increasing, small proportion of MRSA also now shows low-level resistance to Vancomycin (the Drug of choice), leading to treatment failure. For the increase cause of such infection in hospitals, at least two mechanisms have been documented[12]. First, antimicrobial-resistant flora may be endemic within the institution and may be transferred to the patient within the hospital setting. Second, a small population of antimicrobial-resistant bacteria that are a part of patient's endogenous flora at the time of hospitalization may emerge under the selective pressure of antibiotics and become the dominant flora.

II. Material and Methods

The soil sample was taken from hospital wastages dumping site. Soil sample was chosen because of higher probability of finding bacterial stains of localized zone mainly obtained from dump hospital wastages which may include medicines, edibles, patient's dressings *etc*, so there might be probability of finding large amount of pathogenic bacteria. The soil sample was taken from Vivekanand Polyclinic and Ram Manohar Lohia hospital, Lucknow, Uttar Pradesh.

The samples were pre-incubated in two flasks-one containing 10.0% antibiotic supplemented with nutrient broth media and other containing only 10.0% antibiotic. Both flasks were incubated for one week at 37ºC. After one week prepared inoculums were serially diluted and spread on NA plates and incubated overnight.

For further characterisation Glucose, maltose, dextrose and mannitol fermentation tests were performed on their respective broth followed by glucose oxidase, nitrate reductase, urease and MRVP tests.

To check their growth potential all the isolates were selected for antibiotic sensitivity tests. Antibiotic sensitivity test was performed by gel diffusion method by taking different concentrations of antibiotics. Antibiotics used were Ofloxacine, Ciprofloxacin, Tetracycline, Norflox, Amoxicillin, Ampicillin, Cefitraixim, Mahacef, Roxithromycine, Odoxyl, Amoxyclave, Uricomycin, zone of inhibition was calculated for different concentrations[13]. Growth optimization tests were performed to check for the growth enhancement and growth inhibitory materials (other than antibiotics).

In test tubes containing distilled water 1% of yeast, beef extract, maltose, lemon juice, blood, peptone, blood, starch and grape juice were taken and optical density was measured after a week.

Then pH optimization tests was performed at 0.2% beef extract with the pH of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and, 10.0 in separate test tubes. After this inoculated the culture in each test tube and incubated at 37 ^{0}C for overnight. To get the more defined pH values suitable for growth, growth of the microbe was further recorded at different pH. We prepared 0.2% Beef extract media with the pH of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, & 8.5 in separate test tubes.

Microbial growth kinetics analysis was performed in nutrient broth media by measuring the optical density. The effects of metals are both stimulatory and inhibitory on microorganisms. This test was performed to check the growth of microbes. 0.1% Cu^{+2}, Pb^{+2}, Mg^{+2}, Ca^{+2} and Fe^{+2} ions in each test tubes was added in 0.2% beef extract and optical density was measured.

Effect of different concentration of metal (Ca^{+2}) ions on growth:

This test was performed for optimization of metal (Ca^{+2}). We prepared 0.2% of Beef extract media in separate test tubes and added 0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.5%, 1.0% in each test tube and inoculated culture MRSRA1102 MH then all test tube were incubated at 37 ^{0}C for overnight. The isolates were also grown on different growth elicitors for studying their growth optimization study conditions. 0.2% Beef extract media in separate test tubes Growth elicitors included 0.1% Maltose, Starch, Dextrose, Sucrose and Glycine.

Isolation of microbes:

When microbes are isolated by serial dilution, we observed more number of colonies when inoculum is supplemented with culture media & less number of colonies obtained when inoculum is supplemented with antibiotics only, but morphology almost similar *i.e.* circular, elevated, whitish yellow or whitish opaque, shown below in figure-1

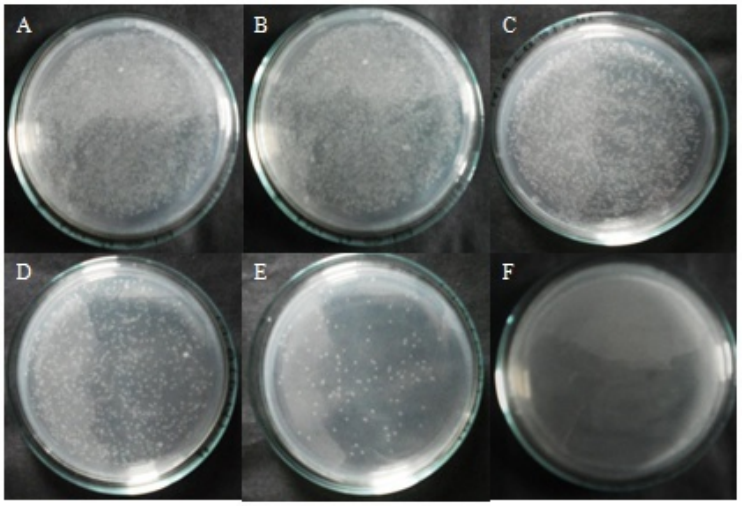

Figure 1: Isolation of microbes. A), B) & C) denoted growth of microbes in culture media supplemented antibiotic solution; D), E) & F) denoted growth of microbes in antibiotic solution only.

Selection of pathogens:

When subculture by streaking method, obtained three types of bacteria and labelled as MRSRA 1101 MH, MRSRA 1102 MH, MRSRA 1103 MH, shown in figure- 2 and were maintained throughout the study.

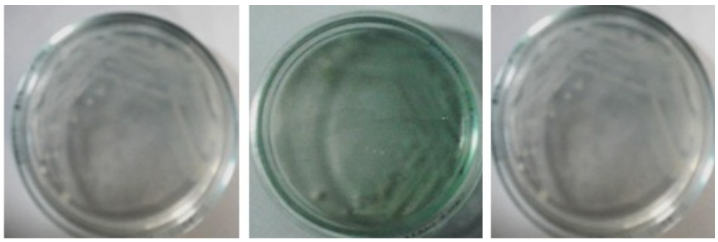

Fig. 2: Pure colonies MRSRA 1101 MH, 1102 MH, 1103 MH

Antibiotic sensitivity test against different antibiotics:

Screening of all the three isolates against various drugs were performed and among all culture, MRSRA1102 was sensitive against Ofloxacin, Ciprofloxacine and Tetracycline, showing the zone of inhibition 2.3, 2.8 and 1.3 cm. respectively (Data shown in Table-1 & figure-3), but this type of culture were resistant against all the antibiotics selected.

Table (1): Antibiotic sensitivity against various antibiotics.

Antibiotics(50µg/ml)	**Zone of inhibition(cm)**
Ofloxacin,	2.30
Ciprofloxacine,	2.80
Tetracycline	1.3
Norflox	0.0
Amoxycillin	0.0
Ampicilin	0.0
Cefitraixim	0.0
Rexi	0.0
Roxithromycine	0.0
Odoxyl	0.0
Amoxyclave	0.0
Uricomycin	0.0

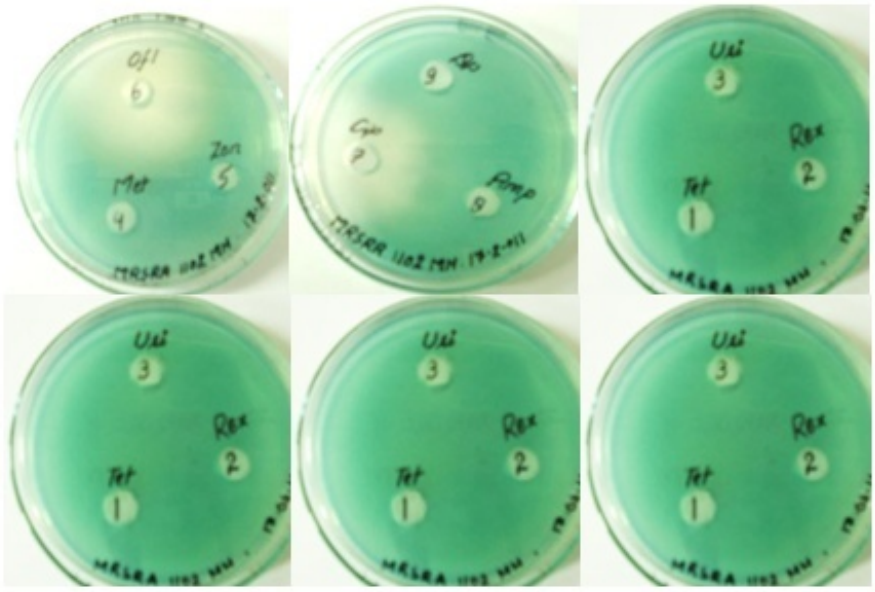

Figure 3: Antibiotic sensitivity against various antibiotics.

Antibiotic sensitivity test against different concentration of antibiotics:

When different concentrations of different antibiotics were screened against isolate MRSRA 1102 MH, growth was inhibited by three antibiotics while other nine antibiotics were unable to check the growth of the microorganism. Data shown below in table-2 & figure-4& 5

Table-(2): Antibiotic sensitivity of MRSRA1102 against various concentrations of antibiotics

Antibiotics	**Concentration (µg/ml)/ Zone of inhibition (in cm.)**						
	50	**100**	**250**	**500**	**750**	**1000**	**1500**
Amoxycilin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ampicilin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Rexi	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cefitriaxime	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Amoxyclave	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ofloxacin	**1.2**	**1.4**	**1.5**	**1.7**	**2.0**	**2.3**	**2.5**
Tetracyclin	**1.3**	**1.5**	**1.8**	**2.2**	**2.5**	**2.7**	**2.9**
Ciprofloxacin	**2.0**	**2.8**	**3.0**	**3.4**	**4.1**	**4.3**	**4.6**
Norflox	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Odoxyl	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Roxithromycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Uricomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0

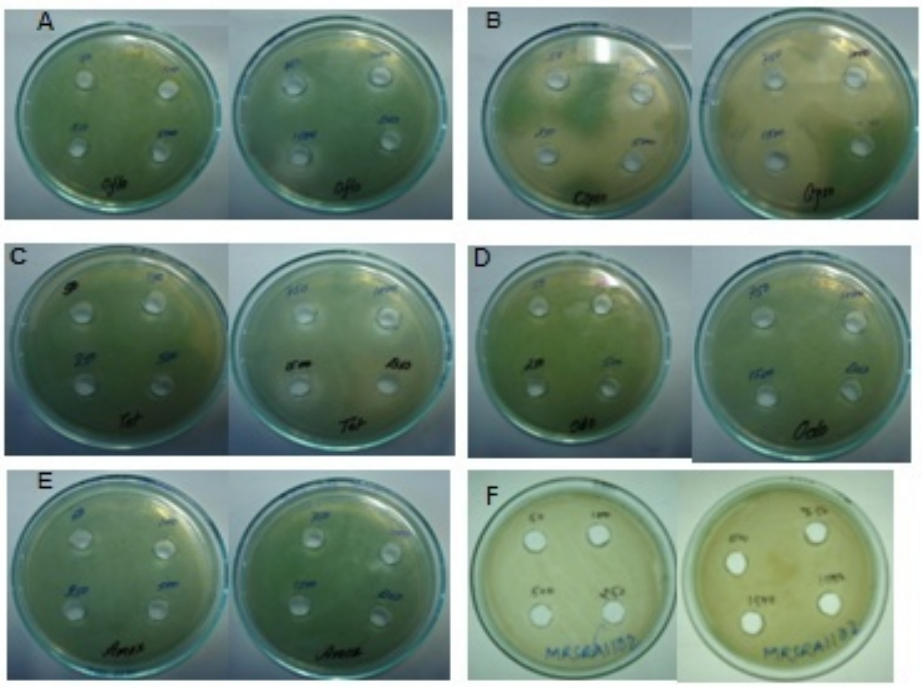

Figure 4: Effect of different antibiotics at various concentrations against isolate MRSRA 1102 MH. A) Ofloxacin, B) Ciproxacin, C) Tetracyclin, D) Metrozyl,E) Amoxyclave, F) Rexi

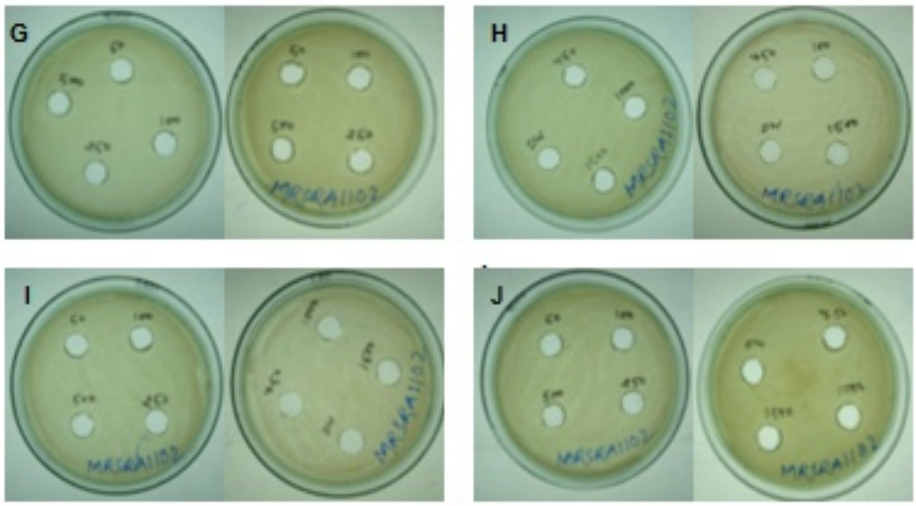

Figure 5: Effect of different antibiotics at various concentrations against isolate MRSRA 1102 MH G) Cefixime, H) Ampicillin, I) Odoxyl, J) Amoxycillin

Identification and Morphological Characterisation of Pathogens:

We observed colony morphology on the basis of margin, surface texture, elevation, optical feature & pigmentation showing shown below in table-3.

Table-(3): Identification and Characterisation of Pathogens.

Colony morphology	**MRSRA1101**	**MRSRA1102**	**MRSRA1103**
Margin	Entire	Entire	Entire
Surface texture	Smooth	Smooth	Rough
Elevation	Elevated	Elevated	Elevated
Optical feature	Slightly convex	Slightly convex	Convex
Pigmentation	Dim light	Greenish	Dim light

Biochemical Tests:

Gram's staining:

After Gram's staining, observed under microscope that MRSRA1102 MH appear purple are referred to as gram positive, *coccus* (on the basis of shape).

Table-(4): Biochemical tests of MRSRA1102

Biochemical test	**Result**
Catalase test	+
Glucose fermentation test	+
Sucrose fermentation test	+
Lactose fermentation test	+
Mannitol fermentation test	-
Endospore staining	-
Gram's staining	+

Study of growth kinetics:

Optical density (O.D) of broth culture of isolate was recorded at the difference of 1hr incubation time and data has been shown in table-5 & figure-6. We plotted a graph between time & O.D, obtained sigmoidal growth curve, showing the four typical phases of growth i.e. A) Lag phage, B) Exponential phase, C) Stationary phase & D) Death phase.

Table-(5): optical density of broth culture of MRSRA1102 MH.

Culture Incubation time(hr)	Optical density at 600nm
1	0.001
2	0.002
3	0.003
4	0.021
5	0.115
6	0.421
7	0.912
8	1.241
9	1.134
10	1.115
11	1.110

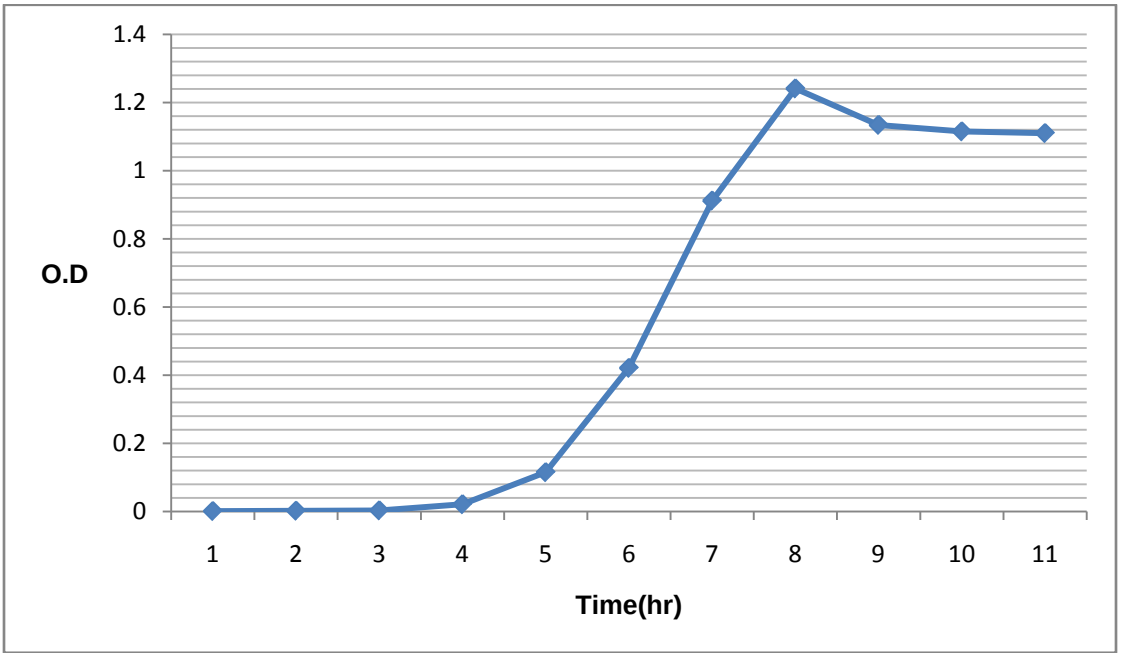

Figure 6: Growth curve of MRSRA1102 MH between O.D & time.

Effect of different media components on growth:

We evaluated nutritional requirement for MRSRA1102 MH & observed that maximum growth occurred in yeast extract but maximum pigmentation occurred in Beef extract media shown in table-6.

Table-(6): Evaluation of nutritional requirement:

Media components (1%)	Growth	Pigment
Starch	+	+
Yeast extract	++++	++
Beef extract	+++	++++
Trypton	++	+

Maltose	--	--
Grape juice	--	--
Blood	--	--

Effect at different concentration of media component on growth:

We evaluated media optimisation at different concentration, observed that maximum growth occurred at 2%, of yeast extract while in beef extract maximum growth occurred at 5%, but maximum pigmentation occurred at 0.2% of yeast extract & 0.1% of beef extract media shown in table-7 & figure-7.

Table-(7): Effect at different concentration of media component:

% of media component	O.D. at 600 nm.		pigment	
	Yeast extract	Beef extract	Yeast extract	Beef extract
0.1	0.545	o.268	++	+++
0.2	0.976	0.442	++++	++
0.5	1.141	0.508	++	_
1.0	1.326	0.821	+	_
2.0	1.516	1.165	_	_
5.0	1.378	1.846	_	_
10.0	0.590	1.578	_	_

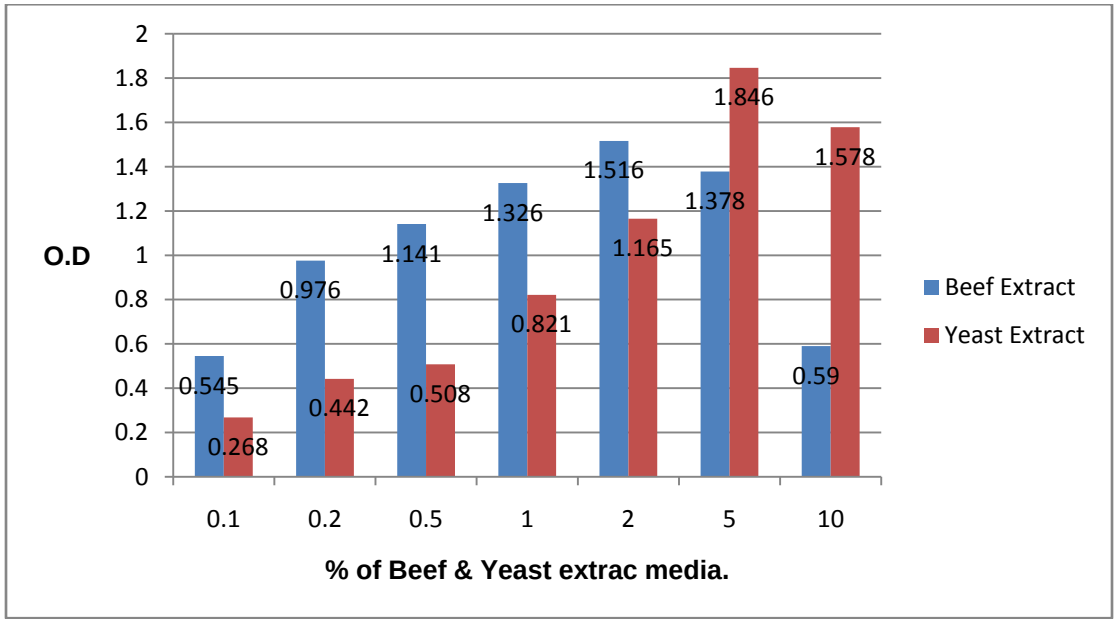

Figure 7: Growth pattern of MRSRA1102 MH between O.D & different % of Beef & extract media

Effect of pH on growth:

We recorded the effect of pH on growth of microbes & observed that maximum growth occurred at pH 8(O.D=0.393) Shown in table-8 & figure-8.

Table-(8): Growth of MRSRA1102 at different PH.

PH of media(0.2% Beef extract)	O.D at 600 nm
4.0	0.150
5.0	0.172
6.0	0.310
7.0	0.359
8.0	0.393
9.0	0.295
10.0	0.125

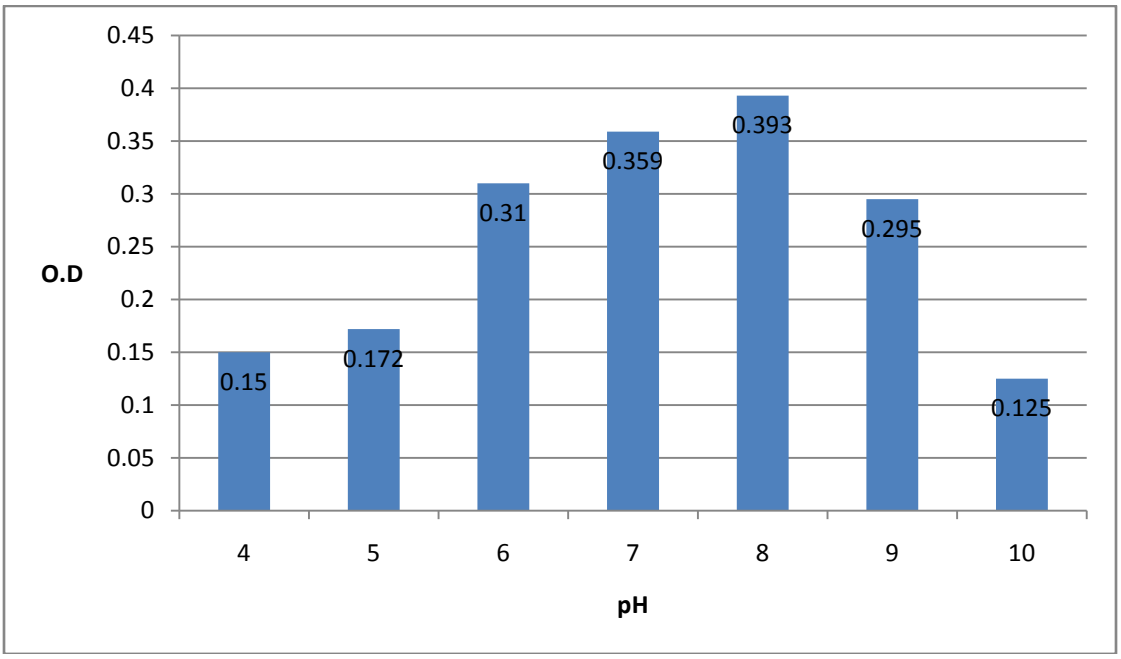

Figure. 8: Growth pattern of MRSRA1102 MH between O.D & pH.

To get the more defined pH value, suitable for growth, growth of the microbe was further recorded at different pH, maximum optical density was observed at pH 7.5, shown below in table-9 & figure-9.

Table-(9): Optical density at different pH.

PH of media(0.2% Beef extract)	**O.D at 600 nm.**
5.5	0.125
6.0	0.350
6.5	0.362
7.0	0.369
7.5	0.373
8.0	0.329
8.5	0.191

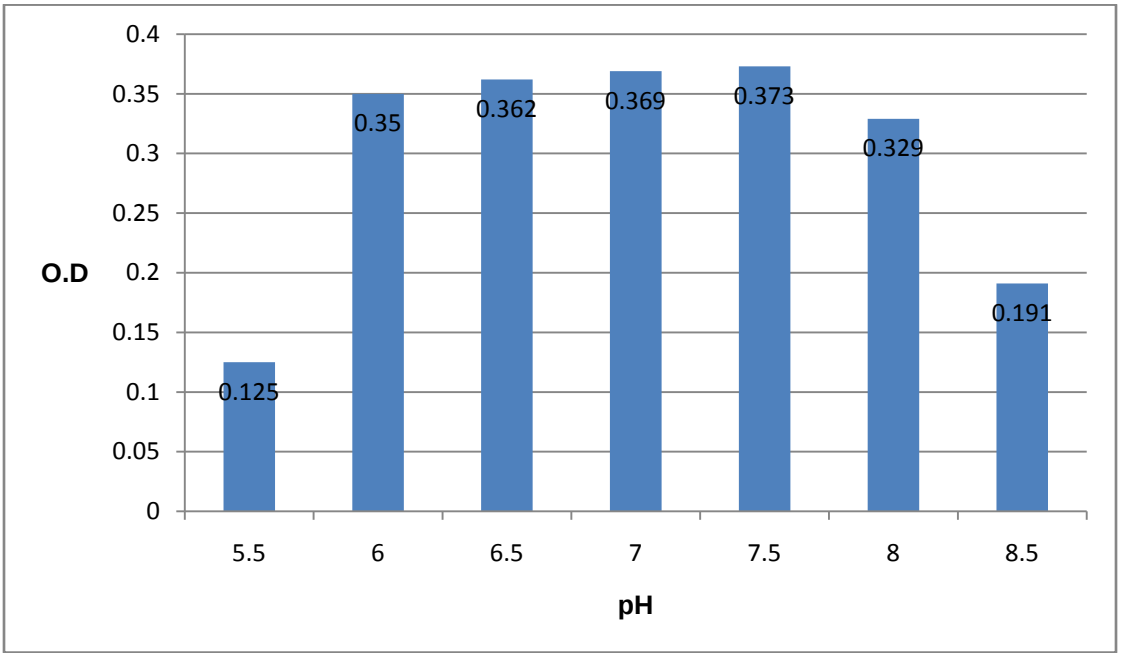

Figure. 9: Growth pattern of MRSRA1102 MH between O.D & pH

Effect of metal ions on growth:

In presence of different metal ions, maximum growth occurred in iron & no growth occurred in copper but maximum pigmentation occurred in calcium ions shown in table-10.

Table-(10): Effect of metal ions:

Metal ions(0.1%)	Growth	Pigment
Cu^{+2}	_	+
Pb^{+2}	+	_
Mg^{+2}	++	++
Ca^{+2}	+++	+++
Fe^{+2}	++++	_
Zn^{+2}	+	_

Effect at different concentrations of metal (Ca^{+2}) ions on growth:

The effect of calcium ions at different concentration, we observed maximum pigmentation occurred at 0.02% as shown below in table-11.

Table-(11): Change in pigment at different concentration of Ca^{+2}.

Concentration of Ca^{+2}(%)	**Pigment**
0.01	++
0.02	++++
0.05	+++
0.1	+++
0.2	++
0.5	++
1.0	+

Effect of elicitors on growth:

The Effect of elicitors on growth of MRSRA1101 was observed that maximum growth occurred in starch & least growth occurred in Glycine, as shown below in table-12.

Table-(12): Effect of elicitors on growth of MRSRA1102.

Elicitors	Growth
Maltose	++
Starch	++++
Dextrose	++
Sucrose	++
Glycine	_

Designing new minimal media for organism:

After one week of incubation in minimal culture media, we observed sufficient growth of MRSRA1102 MH; this broth culture was taken for further proceeds to extraction & purification of intracellular & extracellular bioactive compounds

Extraction and purification of intracellular & extracellular bioactive compound:

We obtained bioactive compound that maintain their growth and susceptibility in the changing environmental conditions. some secrete antibiotics, some secrete growth signals and some mineral degrading enzyme in order to opt for proper nutrition and growth.

Effect of extracellular bioactive compound against various pathogens:

Screening of extracellular bioactive was sensitive against various pathogens & zone of inhibition shown below in table-13 & figure-10.

Table-(13): Effect of extracellular bioactive compound against various pathogens:

Test of organisms.	Zone of inhibition(cm)
E. coli	0.8
Pseudomonas aeruginosa	1.0
Staphylococcus aureus	1.6

Figure. 10: Effect of extracellular bioactive compound against various pathogens .

Effect of Intracellular bioactive compound against various pathogens:

Screening of extracellular bioactive was sensitive against various pathogens & zone of inhibition shown below in table-14 & figure-11.

Table-(14): Effect of Intracellular bioactive compound against various pathogens.

Test of organisms	Zone of inhibition (cm)
E. coli	1.6
Pseudomonas aeruginosa	1.4
Staphylococcus aureus	1.6

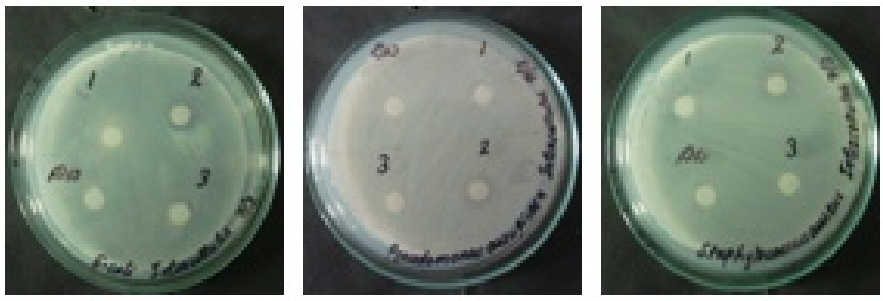

Figure. 11: Effect of Intracellular bioactive compound against various pathogens

III. RESULTS AND DISCUSSIONS

While pre-treatment of soil sample data shows an increase number of colonies for every dilution recorded, when solution was supplemented with nutrient broth, while less number of colonies in antibiotic solution. The shape of colonies, texture, colour was found uniform for both the solution. The three isolates purified are having different characteristics respect of rate of growth, pigmentation & fluorescence respectively.

MRSRA1102 MH was resistant among all the antibiotics screened except Ofloxacine, Ciprofloxacine & Tetracycline respectively at every concentration from 50 to 1500µg/ml, thus having a strong property of multidrug resistant.

Morphological & Biochemical test performed to identify the microbes, the isolates posses varying properties (Table-3,4,5). When growth kinetics was performed, a sharp increase in growth was recorded after 5 hrs inoculation & stabilised in next three hrs. Among several media components tried, yeast extract was best for growth while pigmentation was maximum in beef extract (Table-6). Yeast extract proved direct relation for growth & pigmentation at lower concentration while vice versa in beef extract at same concentration (Table-8).

The growth of microorganism was optimum at pH 7.5, 0.02% calcium ions & 1% starch(Table-9,11&12). All the three isolates possesses very high level of drug resistivity (upto 1500?g/ml) against various drug (12 drugs). MRSRA 1102 MH having too much inhibitory property for different bacteria (E. coli, S. aureus, P. aeruginosa) and fungi (A. niger, Microsporum, C. albicans, T. rubrum) (Table-13,14). MRSRA 1102MH have the capability to produce bluish greenish pigmentation

Characterization of all isolates gave surprising results showing the emergence of *Nisseria* and *Alkaligenes* spp. as MDR pathogens which are generally not. The cause of increasing resistance among the bacteria might be due to development of MDR efflux pump against that drug due to its prolonged exposure at contaminated hospital dumping sites, due to mixing of both MDR and non MDR strains of pathogens at hospital waste disposal site resulting in genetic recombination of plasmids between two bacteria thriving at same place one of which might be MDR or induction of multi drug resistance by proteins secreted by MDR bacteria[14-17]. Antibiotic such as Ciprofloxacin, Ofloxacin belonging to Quinilone family are considered to be best medicines in case of MDR infections and have broad spectrum effects[18-19]. As per the working mechanism it was observed that drug directly dealing with DNA replication *i.e.* DNA Gyrase inhibition by ciprofloxacin are more potent and effective and are less proned to development of resistance by bacteria unless there is development of MDR efflux pump to that drug unlike inhibition of protein synthesis as done by tetracycline or cell membrane disruption by penicillin family of drugs[20-21]. Protein source supplement like beef extract, yeast extract are the best source required for bacterial growth and proliferation, even they can support growth without any other additional nutrient supplement required. Bacteria usually produce its secondary metabolite in stress conditions in order to survive[22-23]. Carbohydrate, amino acids, multivitamin capsules *etc* and metal ions acts as growth elicitors in case of many bacterial strains. The place of emergence of new and pathogenic strains of MDR bacteria can be hospital itself if not taken care of hospital dumped wastages.

IV. CONCLUSION

Hospitals including patient ward, operation theatre and ICU are a potent platform for spreading of pathogens among individuals either healthy or unhealthy persons also there is a maximum exposure of pathogens against various drugs. The bacterial isolates purified from those hospital samples showed more resistance against drugs in comparison to microbes isolated from normal soil samples. Also, bacteria group such as *Nisseria* and *Alkaligenes* spp. were found to exhibit drug resistance isolated from hospital samples redirects the attention of being drug resistant gradually and more vulnerable and pathogenic while growing along with drug resistant microbes, opening a fresh debate of possibility of horizontal transmission of drug resistant characters among organisms. The detailed study of horizontal inheritance of transfer of gene responsible for drug resistance will help us to find a right way in the direction of preventing any microbe not to become drug resistant against any drug.

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