Research Journal of Recent Sciences ISSN 2277-2502
Vol. **2(ISC-2012),** 124-129 **(2013)** *Res. J. Recent. Sci.*

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

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Available online at: www.isca.in
Received 14th September 2012, revised 19th January 2013, accepted 02nd February 2013

Abstract

Soil samples from two different city hospitals were collected, pre-treated along with several antibiotics for primary screening of numerous microbes and 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 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, *Acinetobacter baumannii*, drug resistance.

Introduction

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.

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 ofloxacin, ciprofloxacin, cefitriaxine, mahacef, roxithromycin, norflox, amoxicillin, ampicillin, odoxil, amoxyclav, uricomycin, tetracycline, 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.

Microbial growth kinetics analysis was performed for all three isolates 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.

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.

Results and Discussion

3 bacterial isolates were obtained. The 1st and 3rd isolate showed latexy mocoid growth while 2nd one showed growth with greenish pigmentation

The 1st and 2nd isolates were identified as Gram negative cocci while third isolate as Gram negative bacilli, all showing positive results for catalase test indicating their aerobic nature

On the basis of following biochemical tests the 1 and 2 isolate were characterised as Gram negative aerobic cocci coming under *Nisseria* species whereas culture 3 was characterized as Gram negative aerobic curved rod coming under *Alkaligene* Sp. Category.

Table-1
Table showing comparative colony morphology of the bacterial isolates from different samples

Colony Morphology	1st isolate	2nd isolate	3rd isolate
Margin	Entire	Entire	Entire
Surface Texture	Rough	Smooth	Smooth
Elevation	Elevated	Elevated	Elevated
Optical Texture	Slightly Convex	Slightly Convex	Slightly Convex
Pigmentation	Dim Light	Greenish	Dim Light

Figure-1
Photographs showing pure cultures of the isolates on NA media after incubating at 37 ^{0}C for 48 h

Table-2
Showing results of different biochemical test on culture 1, 2 and 3

Tests	Culture 1	Culture 2	Culture 3
Glucose fermentation	+	+	+
Glucose oxidase	+	_	_
Dextrose fermentation	+	+	+
Maltose fermentation	+	_	+
Mannitol fermentation	+	_	_
Citrate	+	+	+
Nitrate reduction	+	_	+
Urease	+	+	+
MR	_	_	_
VP	+	+	+

Figure-2
Figure showing zone of inhibition of various antibiotics for first isolate

Figure-3
Figure showing zone of inhibition of various antibiotics for second isolate

Figure-4
Figure showing zone of inhibition of various antibiotics for third isolate

Microbial growth kinetics analysis: After measurement of optical density of all the three isolates it was observed that culture 1 gave the best growth.

Table showing analysis of growth pattern of all the three microbes in a given time interval.

Media optimisation of Isolates: All the three isolates showed optimum growth in beef and yeast extract media no growth was observed in case of lemon juice.

Table showing growth of all the three cultures on various components for growth optimization

Table-3
Table showing zones of inhibition for all the three isolates against different antibiotics

Antibiotics	Culture 1	Culture 2	Culture 3
Ofloxacin	2.40	2.30	0.00
Tetracycline	0.00	0.00	0.00
Mahacef	1.50	1.00	1.10
Ciproflox	2.10	2.80	1.60
Amicacin	0.00	0.00	0.00
Amoxyclav	0.00	0.00	0.00
Amoxycilin	0.00	0.00	0.00
Roxithromycin	0.00	0.00	0.00
Cefatriaxone	0.00	0.00	0.00
Zenflox	0.00	0.00	0.00
Odoxil	0.00	0.00	0.00
Metrogyl	0.00	0.00	0.00

Table-4
Table showing growth pattern of isolates in different media components

S.n	Media (1%)	Mrsra 1102	mrsra 1101	mrsra 1103
1	Starch	+	0	0
2	Yeast extract	+++++	++++	++++
3	Beef extract	++++	++++	++++
4	Tryptone	+++	++	++
5	Maltose	++++	+	+
6	Lemon juice	0	0	0
7	Grape juice	0	+	+
8	Blood	++	N.A	N.A

Table-5
Table showing growth pattern of isolates in different media components

Time(hr)	1st isolate	2nd isolate	3rd isolate
1.	0.001	0.000	0.000
2.	0.101	0.005	0.004
3.	0.216	0.045	0.039
4.	0.315	0.086	0.094
5.	0.384	0.159	0.176
6.	0.409	0.239	0.310
7.	0.415	0.341	0.350
8.	0.429	0.349	0.361
9.	0.431	0.351	0.391
10.	0.435	0.350	0.398
11.	0.430	0.349	0.390

Effect of Metal ions on Growth: In presence of different metal ions, maximum growth occurred in iron and no growth occurred in copper but maximum pigmentation occurred in calcium ions

Discussions: 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 destruction 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]. 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.

Table-6
Effect of metal ions on growth of 3rd isolate

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

Table-7
growth pattern of the third isolate under different elicitors

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

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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