Full Length Article

Isolation and Characterization of Multi-Drug Resistant Cultures from Aquatic Areas

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Abstract

Five MDR cultures of bacteria were isolated from four different locations of Lucknow and Kanpur, respectively. Based upon Gram staining four isolates (A1, A2, C2 and G3) were identified as Gram negative out of which A1 was mixed culture that is cocci + rod and A2 and G3 were Gram negative rods. Culture N was identified as Gram positive mixed culture that is cocci + rod. Based upon the biochemical analysis G3 bacterial culture was identified as *Pseudomonas aeruginosa* (Table 1), for multi-drug resistance (MDR) test seven different antibiotics in different concentration were used. It was observed that A1 bacterial culture showed resistance to amoxycilline (100 µg/ml), cefixime (100 µg/ml), ofloxacin (50 µg/ml), roxithromycin (20 µg/ml), ampicilline (50 µg/ml) and tetracycline (50 µg/ml) while A2 culture was resistant to amoxycilline (50 µg/ml) and cefixime (1 mg/ml). C2 was resistant to amoxycilline (10 mg/ml), cefixime (1 mg/ml), roxithromycin (10 mg/ml), ampicilline (20 µg/ml) and tetracycline (20 µg/ml). G3 was resistant to amoxycillin (1 mg/ml) and cefixime (1 mg/ml). And N culture was resistant to amoxycilline (100 µg/ml), cefixime (1 mg/ml), roxithromycin (50 µg/ml), ampicilline (50 µg/ml) and tetracycline (20 µg/ml).

Keywords: Multi-Drug Resistance (MDR), Susceptibility; Antibiotics; Bacterial Isolates; Broad Spectrum; Minimum Inhibitory Concentration (MIC).

Abbreviations : µg- Microgram; ml- Mililitre; mg - Miligram; R - Resistant; S- Sensitive; AR-Antibiotic Resistance; hrs.-Hours; MBC -Minimum Bactericidal Concentration; MIC- Minimum Inhibitory Concentration; MDR- Multi-Drug Resistance

Introduction

Antibiotic resistance is a considerable term while studying about diseases in human, animals and plants. Mostly the antibiotic resistant bacterial cultures are found in clinical and veterinary land areas whereas the existence of antibiotic resistance (AR) bacteria in water has also observed and supported to be present in higher concentrations and diversity in hospital areas as compared to domestic areas. Antibiotics are either synthesized industrially or also produced by microorganisms; these antibiotics have microstatic or microcidal activity. Mainly these antibiotics producing microbes interrupt the microbial metabolism by various mechanisms (Jalal *et al.,* 2010). Several researches were performed that dealt with advanced antimicrobial resistance in bacteria isolated from food, animal and environment (Jensen *et al.,* 2001). The use of antibiotics is increasing continuously in different fields like veterinary medicine, agriculture, medicines etc. but the awareness of knowledge regarding the quantity of antibiotics present in the environment after their use is very less (Hirsch *et al.,* 1998). The population of antibiotic resistance (AR) bacteria in the areas where antibiotics are used is common but existence of AR bacteria in aquatic environment is also increasing readily (Schwartz *et al.,* 2003). It is observed that resistance in bacteria could be – (i) intrinsic resistance i.e. natural resistance of bacteria to certain antibiotics. (ii) Acquired resistance i.e. the susceptible that become resistant by adapting itself through genetic changes. (iii) Multi--drug resistance i.e. the resistance of bacterium to be effective against MDR bacterial cultures. The microorganisms have a defense system against various antibiotics and they grow even in presence of antibiotics by getting resistant. It was suggested that soil microbes harbour antibiotic resistant (AR) genes with a diverse gene sequencing (Jalal, *et al.,* 2010). The antibiotic sensitivity is expressed in terms of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) and it give quantitative data. These quantitative results are useful in predicting the tissue, blood or urine levels of antibiotics that must be attained to assure inhibition or killing. In comparison to clinical or hospital area soil, the soil from various aquatic places were taken for isolating the AR pathogens. The main objective of this work is to isolate the multi--drug resistant (MDR) pathogens from the aquatic places of Lucknow and Kanpur, U.P against various antibiotics and to know the MIC and MBC values for various antibiotics in presence of the isolated pathogens.

Materials and Methods

The soil samples were collected from five different locations of Lucknow and Kanpur; A (Sewage water, Lucknow), C (Pond water, Lucknow), G (Ganga river, Kanpur) and N (Gomti river, Lucknow). Samples were serially diluted and bacteria were isolated on Nutrient

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Agar, two bacterial colonies were selected and designated as: A1, A2, C1, C2, G1, G2, N1 and N2, respectively. Bacterial colonies were sub cultured several times up to pure culture, maintained for further biochemical analysis, and preserved at low temperature. After isolation, identification of the bacterial isolates was carried out according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbonns, 1978).

All the bacterial isolates were analyzed for their sensitivity against commonly used antibiotics by agar well diffusion method. Different antibiotics having different mechanisms of action were taken as tetracycline, amoxycilline, ampicilline, roxithromycin, ofloxacin, cefixime and ciprofloxacin of concentration 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 50 µg/ml, 100 µg/ml, 1 mg/ml and 10 mg/ml. Wells were prepared on nutrient agar plate previously spreaded with 50 µl of isolated bacterial broth culture. These wells were loaded with 50µl of antibiotic solution and then plates were incubated at 37°C for overnight, then the zone of inhibition were examined. The obtained multi-drug resistant isolates were further characterized by microscopic and Biochemical studies. The purified cultures were characterized by staining (Gram staining and Endospore staining) and biochemical activity (IMVIC) as par Bergey's manual given in Aneja (2003). King's B medium is a confirmation medium for detection and differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* based on fluorescein (pyoverdin) production and pyocyanin inhibition. Peptone, potassium hydrogen phosphate, heptahydrated magnesium sulfate ($MgSO_4.7H_2O$) and bacteriological agar were added to distilled water (pH 7.0 – 7.2). Solution was autoclaved and poured in test tube to prepare slants. The isolates were inoculated on slants and kept for incubation at 37°C for 48 hrs.

MIC is ``the lowest concentration which resulted in maintenance or reduction of inoculum viability". Only antibiotic that shows inhibitory activity toward the bacterial isolate using the Kirby-Bauer methods are tested further. The active antibiotics were serially diluted to make a range of antibiotic concentrations that encompasses the concentration used in the (Kirby-Bauer method, 1966). 20 µl of test bacterium was added to all tubes having 3 ml of nutrient broth which was serially diluted with antibiotic. After incubation, the MIC is identified as the least concentration of antibiotic that inhibits the growth of the test bacterium. The Minimal Bactericidal Concentration (MBC) is determined by spreading the bacterial colonies on nutrient agar plate which was obtained by MIC.

Biochemical tests	Isolate A1	Isolate A2	Isolate C2	Isolate G3	Isolate N
Gram's stain	-	-	-	-	+
Cellular morphology	Rods +cocci	Rods	Rods	Rods+cocci	Rods+cocci
Catalase activity	+	+	+	+	+
Methyl-red test	-	+	-	-	-
Voges proskauer's test	+	-	+	+	+
Citrate utilization	+	- (Family Enterobacteriaceae)	+	+	+
King's B medium test	-	-	-	+ *(Pseudomonas aeruginosa)*	-
Endospore stain	-	-	-	-	+

Table 1. Biochemical analysis of isolated MDR bacterial cultures.

Concentration (µg/ml	Amoxy cilline	Cefixime	Ofloxacin	Roxithromicin	Ampicillin	Tetracycline	Ciprofloxacin
5	R	R	R	R	R	R	S
10	R	R	R	R	R	R	S
20	R	R	R	R	R	R	S
30	R	R	R	S	R	R	S
50	R	R	R	S	R	R	S
100	R	R	S	S	S	S	S
1 (mg/ml)	S	R	S	S	S	S	S
10 (mg/ml)	S	S	S	S	S	S	S

Table 2. MDR test for sample A1 [*Note:* R= resistant, S= sensitive].

Concentration (µg/ml	Amoxy cilline	Cefixime	Ofloxacin	Roxithromicin	Ampicillin	Tetracycline	Ciprofloxacin
5	R	R	S	S	S	S	S
10	R	R	S	S	S	S	S
20	S	R	S	S	S	S	S
30	R	R	S	S	S	S	S
50	R	R	S	S	S	S	S
100	R	R	S	S	S	S	S
1(mg/ml)	R	R	S	S	S	S	S
10 (mg/ml)	S	S	S	S	S	S	S

Table 3. MDR test for sample A2 [Note: R= resistant, S= sensitive].

Results

Five MDR cultures of bacteria were isolated from four different locations of Lucknow and Kanpur. Based upon Gram staining, four isolates (A1, A2, C2 and G3) were identified as Gram negative out of which A1 was mixed culture i.e. cocci + rod and A2 and G3 were Gram negative rods. Culture N was identified as Gram positive mixed culture that is cocci + rod. All isolated bacterial cultures were aerobic. Based upon the biochemical analysis, G3 bacterial culture was identified as *Pseudomonas aeruginosa* (Table 1). For the multi- drug resistance

Concentration (µg/ml	Amoxy cilline	Cefixime	Ofloxacin	Roxithromicin	Ampicillin	Tetracycline	Ciprofloxacin
5	R	R	S	R	R	R	S
10	R	R	S	R	R	R	S
20	S	R	S	R	R	R	S
30	R	R	S	R	S	S	S
50	R	R	S	R	S	S	S
100	R	R	S	R	S	S	S
1(mg/ml)	R	R	S	R	S	S	S
10 (mg/ml)	R	S	S	R	S	S	S

Table 4. MDR test for sample C2 [Note: R= resistant, S= sensitive].

Concentration (µg/ml	Amoxy cilline	Cefixime	Ofloxacin	Roxithromicin	Ampicillin	Tetracycline	Ciprofloxacin
5	R	R	S	S	S	S	S
10	R	R	S	S	S	S	S
20	S	R	S	S	S	S	S
30	R	R	S	S	S	S	S
50	R	R	S	S	S	S	S
100	R	R	S	S	S	S	S
1(mg/ml)	R	R	S	S	S	S	S
10 (mg/ml)	S	S	S	S	S	S	S

Table 5. MDR test for sample G3 [Note: R= resistant, S= sensitive].

Concentration (µg/ml	Amoxy cilline	Cefixime	Ofloxacin	Roxithromicin	Ampicillin	Tetracycline	Ciprofloxacin
5	R	R	S	R	R	R	S
10	R	R	S	R	R	R	S
20	S	R	S	R	R	R	S
30	R	R	S	S	R	S	S
50	R	R	S	R	R	S	S
100	R	R	S	R	R	S	S
1(mg/ml)	R	R	S	S	S	S	S
10 (mg/ml)	S	S	S	S	S	S	S

Table 6. MDR test for sample N [Note: R= resistant, S= sensitive].

Test tubes	O.D at 600 (nm)	Conc.(mg/ml)
1	0.52	0.25
2	0.72	0.06
3	0.85	0.015
4	0.92	0.0037
5	0.99	0.00093
6	1.20	0.000023

Table 7. MIC value of culture A2 against Cefixime
(Initial concentration= 1mg/ml)

(MDR) test seven different antibiotics in different concentration were used. It was observed that A1 bacterial culture showed resistance to amoxicilline (100 µg/ml), cefixime (100 µg/ml), ofloxacin (50 µg/ml), roxithromycin (20 µg/ml), ampicilline (50 µg/ml) and tetracycline (50 µg/ml). A2 culture was resistant to amoxicilline (50 µg/ml) and cefixime (1 mg/ml). C2 was resistant to amoxicilline (10 mg/ml), cefixime (1 mg/ml), roxithromycin (10 mg/ml), ampicillin (20 µg/ml) and tetracycline (20 µg/ml). G3 was resistant to amoxicilline (1mg/ml) and cefixime (1 mg/ml). And N culture was resistant to amoxicilline (100 µg/ml), cefixime (1 mg/ml), roxithromycin (50 µg/ml), ampicilline (50 µg/ml) and tetracycline (20 µg/ml). It was observed that even after showing resistance at higher concentrations amoxicilline was sensitive at concentration 20 µg/ml against four cultures (A2, C2, G3 and N) which was considered as MIC value of amoxicilline. Roxithromycin also showed sensitivity at concentration 30 µg/ml against culture N which was considered as its MIC value.

Discussion

Antibiotics are chemotherapeutic agents that have revolutionized the treatment of infectious disease – turning life-threatening diseases into more manageable and treatable conditions. Resistance became a major challenge to the treatment of infectious diseases shortly after the introduction of antibiotics. The objective of the experiment was to isolate multi-drug resistant bacteria which can use antibiotics as carbon source for their nutrition and growth. Bacteria gain resistance through various methods: some bacteria make an antibiotic ineffective before the drug can kill them; some strains alter the drug attack site so that the antibiotic becomes ineffective; some rapidly pump out the antibiotic–antibiotic efflux. Some bacteria have a natural resistance to antibiotics but others become resistant through genetic mutation or by acquiring resistance from another bacterium (Alliance for the Prudent Use of Antibiotics, 1999). Five multi-drug resistant bacterial cultures were isolated from all sampling points. Out of which only one was identified as *Pseudomonas aeruginosa*. This culture causes infections of wounds, burns, eyes and ears (Lateef, 2003). The antibiotic sensitivity pattern of the five bacterial isolates is shown in Tables (2–6). All five cultures were resistant to cefixime and amoxycillin even at higher concentrations (100 µg), which was much higher compare to work done by Lateef (2003) this process was done with the help of Agar well diffusion method which was earlier done by Bauer *et al.* (1966). Only A1 isolate was resistant to ofloxacin.A1, C2 & N isolates were resistant to roxithromycin, ampicillin and tetracycline. None of the five isolates were resistant to ciprofloxacin i.e. all were susceptible to ciprofloxacin. After MIC the antibiotics amoxycillin, cefixime showed resistance at higher concentration (0.25 mg/ml). After MBC it was observed that the MDR

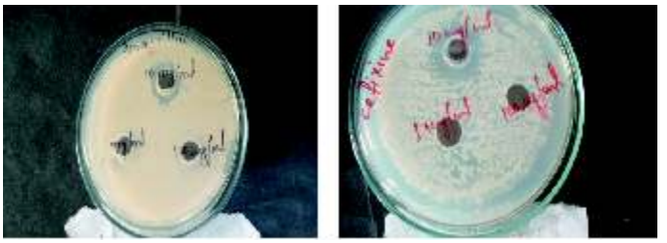

Figure 1. Resistance shown by isolated MDR culture C_2 at higher concentration (100 µg/ml, 1 mg/ml & 10 mg/ml) of cefixime and amoxycilline

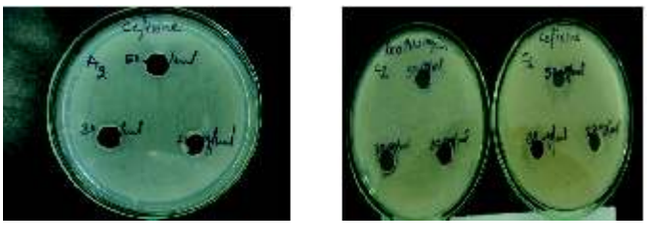

Figure 2. Resistance shown by MDR isolates at lower concentrations of cefixime and roxithromycin for cultures A2 & C2

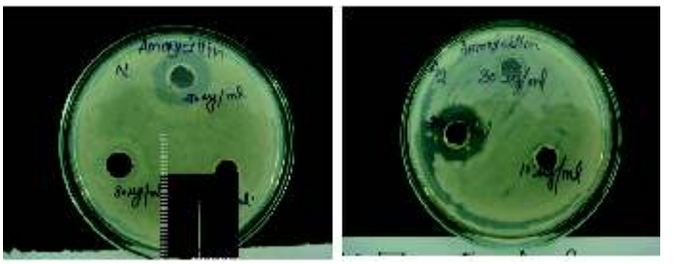

Figure 3. Resistance shown by isolates N & A2 with amoxycilline

Figure 4. MBC culture plates of the isolates (Cultures showing the growth in the presence of Antibiotics) A2: culture against Cefixime C2: culture against Amoxycilline

Figure 5. Confirmatory test of *Pseudomonas aeruginosa* in G3 isolate (Pink colonies in King's B Media).

Test tubes	O.D at 600 (nm)	Conc.(mg/ml)
1	0.45	0.25
2	0.65	0.06
3	0.75	0.015
4	0.92	0.0037
5	0.97	0.00093
6	1.05	0.000023

Table 8. MIC value of culture C2 against Cefixime (Initial concentration= 1mg/ml)

Test tubes	O.D at 600 (nm)	Conc.(mg/ml)
1	0.50	0.25
2	0.65	0.06
3	0.75	0.015
4	0.86	0.0037
5	0.96	0.00093
6	1.10	0.000023

Table 9. MIC value of culture C2 against Amoxycilline (Initial concentration= 1mg/ml)

Test tubes	O.D at 600 nm	Conc.(mg/ml)
1	0.32	0.25
2	0.45	0.06
3	0.56	0.015
4	0.75	0.0037
5	0.85	0.00093
6	0.96	0.000023

Table 10. MIC value of culture A2 against Amoxycilline (Initial concentration= 1mg/ml)

cultures are bacteriostatic as they show growth even at higher concentrations of antibiotics.

Conclusion

At the end of all the experiments it was identified that out of all bacterial cultures isolated from various aquatic places, five were multi--drug resistant. Isolates A2 & C2 are Gram negative rods, isolates A1 & G3 are Gram negative mixed cultures and only one isolate N is Gram positive mixed culture, all isolates were catalase positive. Isolate G3 was identified as *Pseudomonas aeruginosa* and A2 of family *Enterobacteriaceae*. All these five isolates were multi--drug resistant as they were resistant to various antibiotics used which were of different mechanism of action. After MIC & MBC tests it was observed that the isolated cultures were bacteriostatic in nature.

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