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Isolation and Characterization of Multi Drug Resistance Cultures from Waste Water

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Abstract: The antibiotic sensitivity tests were performed for the isolated cultures obtained from hospital waste and laundry water in Lucknow. During the study, out of 16 cultures, 3 bacterial isolates were identified with the help of Bergey's manual. They included; *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus* species. The 6 antibiotics were used *Amoxicillin, Ampicillin, Tetracycline, Ofloxacin, Chloramphenicol, Ciprofloxacin* at lower to higher conc. (10µg-10mg). The best results obtained for *Ofloxacin, Ampicillin, Chloramphenicol* antibiotics (10µg-1mg). The resistance of the 3 bacterial isolates to the commonly used antibiotics revealed that for all antibiotics, all the cultures were showing resistance and against *Ofloxacin,* it was 100% and for *Chloramphenicol,* the resistance activity was measured 80%. The MIC and MBC were also performed for identified cultures.

Key words: Antibiotic sensitivity test, Resistance, MIC and MBC.

Introduction:

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]. *Bacillus sp*, *Staphylococcus sp*. and *Streptococcus* species are bacteria frequently encountered in hospital wastewater, varying between 5 and 10%, *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* have also been reported along with varying numbers of other nosocomial pathogens such as *Klebsiella, Proteus* and *Enterobacter* species [2].Most of these 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 [3].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 [4].The widespread and often in-appropriated administration of antibiotic in livestock, pets, and humans has been shown to result in the development of antibiotic-resistance bacteria and is generally accepted to be the primary pathway for proliferation of antibiotic-resistant bacteria in the environment [5]. The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogens .There are several routes of entry of antimicrobial agents into the environment. Studies have shown that introduction by these routes has changed the antibiotic susceptibility of the microbes in those environments [3]. Waste effluent from hospitals contains high numbers of resistance bacteria and antibiotic residues at concentration able to inhibit the growth of susceptible bacteria [6].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 [7] showed a decrease in VRE from 16% in untreated wastewater to 12.5% at the outlet. High numbers of resistant coliforms have also been found in treatment plant effluents [8] and rivers receiving effluent from treatment plants have higher numbers of resistance profiles of microorganisms isolated from hospital or community sewage.

The present study is carried out to isolate and characterize the multi-drug resistance (MDR) pathogens from waste water which was obtained from various place of Lucknow and to check the activity of culture in the presence of various antibiotics. MIC and MBC test were done to know the inhibitory concentration of antibiotics against various isolate pathogens and to know the minimum bactericidal concentration of the isolated cultures.

Materials and Methods:

Collection of sample: The water samples were collected from different hospital region as well as wastage region like laundry water of Lucknow. These water samples were designated as sample D2, S2 and W1. Sample D2 = Sample from Divine hospital, Sample S2 = Sample from Sahara hospital, Sample W1 = Sample from dump water. These samples were isolated for bacteriological analysis by serial dilution and then agar plate culture techniques.

Serial dilution:

This method is based on the principle that when soil sample or water sample along with bacterial colonies taken, the result obtained in the form of reduce number of bacterial colonies in order to get pure colonies. The microbes are having importance in the industries for enzyme and antibiotic production.

Characterization of bacterial culture:

Gram staining of bacteria:

The gram-negative bacterial cell wall is thin, complex, multilayered structure and contains relatively a high lipid contents, in addition to protein and mucopeptides. The higher amount of lipids is readily dissolved by alcohol, resulting in the formation of large pores in the cell wall which do not close appreciably on dehydration of cell wall proteins, thus facilitating the leakage of crystal violet-iodine (CV-I) complex and resulting in the decolorization of the bacterium which later takes the counter stain and appears red. In contrast, the gram positive cell walls are thick and chemically simple, composed mainly of protein and cross-linked mucopeptides. When it was treated with alcohol, it causes dehydration and closure of cell wall pores, thereby not allowing the loss of (CV-1) complex and cells remain purple [9].

Make a thin smear of culture on glass slides, dried the smear and heat fix, cover the smear one by one with crystal violet (60 seconds), gram's iodine (60 seconds), 95% C_2H_5OH (20 seconds) and safranin (40 seconds). Air dried the slides after washing with D/W and observed under microscope.

Endospore staining:

Some bacteria are capable of changing into dormant structures that are metabolically inactive and do not grow or reproduce. These structures are called as endospores. An endospore develops in a characteristic position with a cell i.e. either central, sub-terminal or terminal.

Thin smears of bacterial isolates and *Staphylococcus aureus* (taken as ideal) were prepared on clean glass slides which were then flooded with 5% malachite green solution. The slides were heated to steaming for about 5 minutes then slides were washed with distilled water then counter stained with 0.5% safranin for 30 seconds and again washed with distilled and observed under microscope.

Biochemical method was performed to differentiate the unknown cultures and for this catalase test, carbohydrate test, citrate utilization test and methyl red voges proskauer test were performed according to process given by [9].

Confirmatory test for Pseudomonas aeruginosa using king's B medium:

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 sulphate and bacteriological agar were added to distilled water (pH 7.0-7.2). Solutions were autoclaved and then poured in test tubes to prepare slants. The isolates were inoculated on slants and kept for incubation at 37°C for overnight.

Confirmatory test for MRSA (Methicillin resistance S. aureus):

Prepare N.B solution, after autoclaving add 1% Methicillin or cloxacillin and inoculate bacterial culture and incubate at 37°C for 24-48 hours. Observed result in the form of bacterial culture growth.

Confirmatory test for Bacillus culture using starch hydrolysis test:

Starch is a complex carbohydrate (Polysaccharide) composed of two constituents- amylose, a straight chain polymer of 200-300 glucose units and amylopectin a larger branched polymer with phosphate groups. Melt the starch agar medium, cool to 45°C and pour into the sterile petri dishes. Allow it to solidify. Label each of the starch agar plate with the name of the organism to be inoculated. Using sterile technique, make a single streak inoculation of each organism into the centre of its appropriately labeled plate. Incubate the bacterial inoculated plates for 48 hours at 37°C. Flood the surface of the plates with iodine solution with a dropper for 30 seconds. Pour off the excess iodine solution. Appearance of golden color zone showed positive test.

Antibiotics sensitivity test:

It is the method to check sensitivity of antibiotics, if cultures will be resistant for antibiotics then show the growth and if antibiotic is sensitive then they will inhibit the growth of cultures and result can be seen in the form of zone of inhibition. This method was based on agar well diffusion method [10].

Prepare N.A plates and spread 50µl of isolated culture. Prepare wells and load antibiotics of lower to higher conc. then Incubate at 37° C for over night and observed results.

MIC (Minimum inhibitory concentration):

The least conc. of antibiotics which will inhibit the growth of pathogens are known as MIC [11] and this method was done with micro-broth dilution method. Prepare nutrient broth and add 1 ml antibiotic of 1 mg concentration in first test tube and mixed properly and transferred 1 ml in second test tube and so on. In last test tube discard same amount of solution. After this step, add 20µl of the pathogen. Incubate at 37°C in shaker incubator. Observed result and take OD at 600 nm.

MBC (Minimum Bacteriocidal Concentration):

The bacterial culture showed the growth in the form of bacteriostatic and bacteriocidal. Bacteriostatics showed the constant growth of culture and bacteriocidal showed lethal conc. of bacterial culture.

Prepare N.A. plates and spread 20µl of MIC tube cultures according to their arrangement. Incubate at 37°C for over night. Observed result in the form of bacterial colonies.

Results:

A total of 16 cultures of bacteria were isolated from 6 different areas of Lucknow and out of 16 isolates there were 3 cultures were detected for their antibiotic sensitivity test. The MIC and MBC tests were also performed.

Serial dilution method:

The serial dilution method was performed in order to get pure and reduce number of bacterial colonies and there were total 16 isolates were found and out of 16 cultures 3 cultures were used for further work.

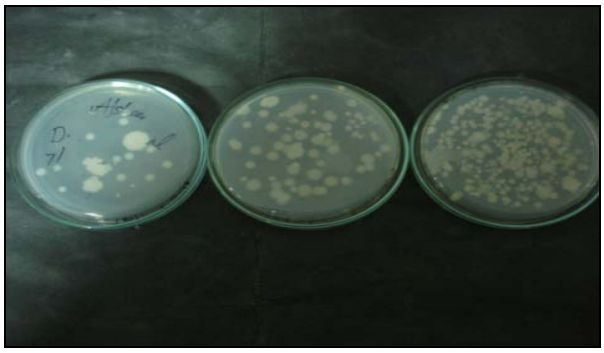


Figure 1: Bacterial colonies in a mixed culture form

Figure 1 showed that mixed cultures in the form of bacterial colonies.

Colony morphology:

Table 1: **Colony morphology of D2, S2, W1**

Characteristics	D2	S2	W1
Shape	Circular	Filamentous	Circular
Elevation	Convex	Flat	Flat
Pigmentation	Yellowish	Off white	Off white
Margin	Entire	Discrete	Entire
Surface	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque

Table 1 **showed that the colony morphology of all the 3 cultures.**

Table 2: **Biochemical analysis of isolated MDR bacterial cultures**

Bio-chemical Test	Isolate D2	Isolate S2	Isolate W1
Gram stain	Gram+ ve rods	Gram- ve rods	Gram + rods
Endospore stain	+	-	-
Catalase test	-	+	+
Bio-chemical test			
Carbohydrate test	+	+	+
Citrate utilization test	-	-	+
MPVP test	-	-	-

Table 2 showed that the biochemical test results for all the 3 cultures and (+) indicate positive result and (–) indicate negative result.

Figure 2: **Catalase test Figure 3 showed that bubbles indicating positive result**

Confirmatory test:

1. Kings' B Media is used to check the presence of Pseudomonas aeruginosa:

Figure 3**: Growth of S2 (*Pseudomonas aeruginosa)* in King's B media**

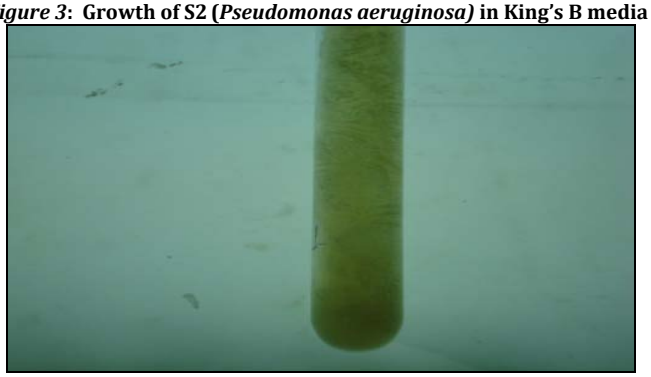
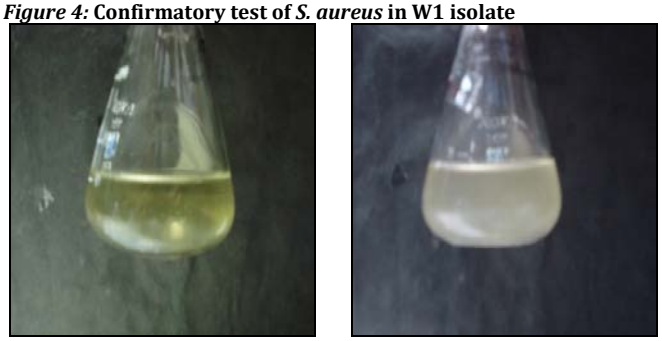


Fig 3 showed the presence of *Pseudomonas aeruginosa* in the form of S_2 culture.

2. Methicillin resistant Staphylococcus aureus (MRSA):

Figure 4: **Confirmatory test of *S. aureus* in W1 isolate**



(a) N.B media with methicillin (b) Growth of *S. aureus* in media with methicillin

Figure 4 showed that MRSA (methicillin resistant *S. aureus*) in the form of W1 isolate in the presence of Methicillin antibiotic at higher conc. (2mg/ml).

(3) Starch hydrolysis test:

To determine the absence or presence of starch in the medium by using iodine solution as an indicator.

Figure 5: **Confirmatory test of *Bacillus* in D2 isolate**

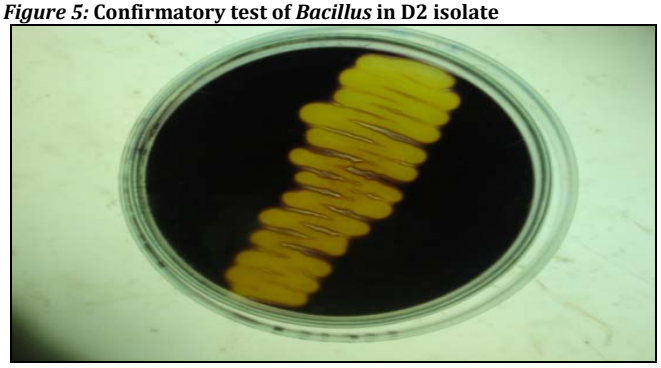


Fig 5 showed that the zone (yellow) showed that culture was hydrolyzing starch which indicated that it was *Bacillus*.

Antibiotics sensitivity test:

If the cultures were showing growth in the presence of antibiotics means cultures were resistant for that antibiotic and if antibiotic will inhibit the growth of that culture in the form of zone of inhibition means antibiotic was sensitive.

Figure 6: **Antibiotics sensitivity test for D2 culture**

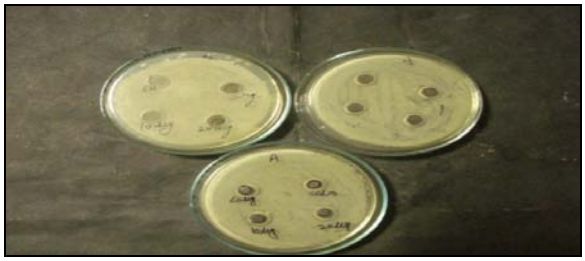


Figure a: Ampicillin

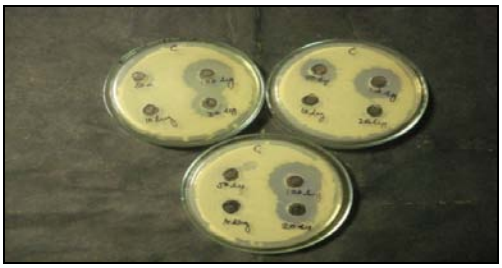


Figure b: Ciprofloxacin

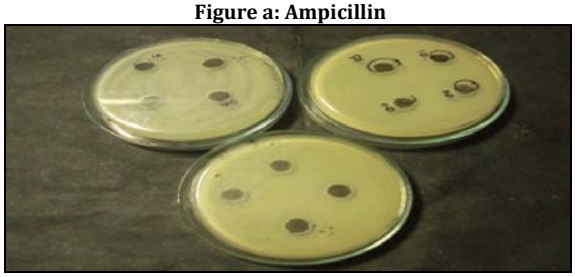


Figure c: Chloramphenicol

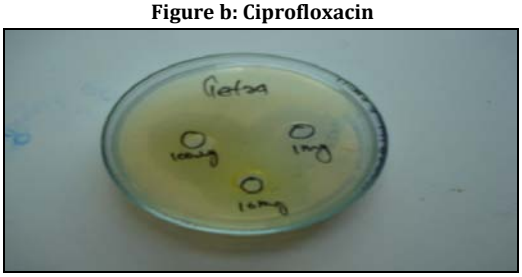


Figure d: Tetracycline

Figure 6 showed that antibiotics were used a *(Ampicillin)*, b *(Ciprofloxacin),* c *(Chloramphenicol),* d (*Tetracycline*) from lower to higher concentration (100µg/ml to 10 mg/ml) ,the culture showed growth in presence of antibiotics till 1 mg/ml concentration but at 10mg/ml the antibiotic was sensitive.

Table 3: **MDR test for D2**

Concentration	Amoxycillin	Ofloxacin	Ampicillin	Tetracycline	Ciprofloxacin	Chloramphenicol
10µg	R	R	R	R	R	R
20µg	S	R	R	S	S	R
50µg	R	R	R	R	R	R
100µg	S	R	R	S	S	R
1mg	S	R	S	S	S	R
10mg	S	R	S	S	S	S

R= Resistance, S= Sensitive

***Table 3*: showed that the 6 antibiotics were used *Amoxicillin, Ofloxacin, Ampicillin, Tetracycline Ciprofloxacin* and *Chloramphenicol* and after getting result it was clear that the culture D2 was showing resistant at higher concentration of *Ofloxacin* and *Chloramphenicol*.**

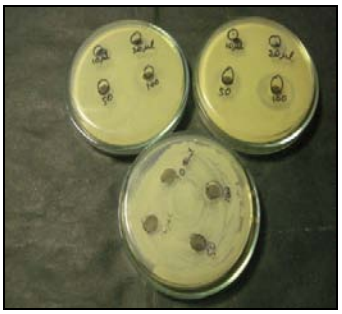


Figure a: Ampicillin

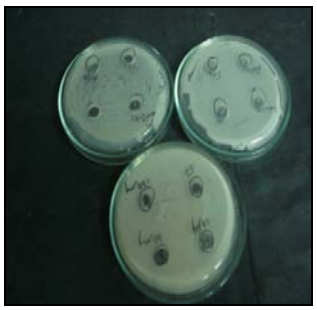


Figure b: Amoxycillin

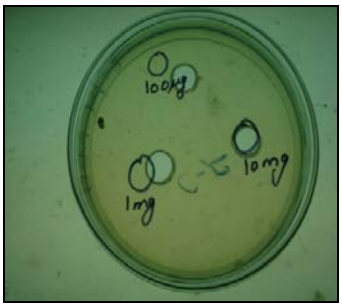


Figure c: Chloramphenicol

Figure d: Ofloxacin

Figure 7: **Antibiotics sensitivity test for S2 culture**

Figure 7 showed that antibiotics were used a *(Ampicillin)*, b *(Ciprofloxacin),* c *(Chloramphenicol),* d (*Tetracycline*) from lower to higher concentration (100µg/ml to 10 mg/ml), the culture showed the growth in presence of antibiotics till 1 mg/ml concentration but at 10mg/ml the antibiotic was sensitive.

Table 4: **MDR test for S2**

Concentration	Amoxycillin	Ofloxacin	Ampicillin	Tetracycline	Ciprofloxacin	Chloramphenicol
10µg	R	R	R	R	R	R
20µg	R	R	R	R	R	R
50µg	R	R	R	S	S	R
100µg	R	R	R	S	S	R
1mg	S	S	S	R	S	R
10mg	S	S	S	R	S	S

R= Resistance, S= Sensitive

***Table 4* showed that the 6 antibiotics were used *Amoxicillin, Ofloxacin, Ampicillin, Tetracycline Ciprofloxacin* and *Chloramphenicol* and after getting result it was clear that the culture S2 was showing resistant at higher concentration of *Tetracycline* and *Chloramphenicol*.**

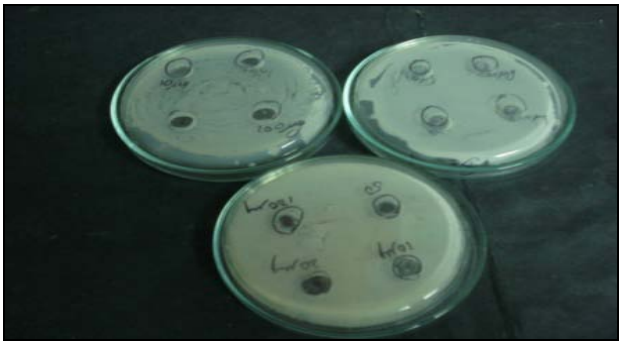


Figure a: Amoxycillin

Figure b: Ampicillin

Figure 8: **Antibiotics sensitivity test**

Figure c: Chloramphenicol

Figure d: Ciprofloxacin

Figure 8 **showed that antibiotics were used a** ***(Ampicillin)*****, b** ***(Ciprofloxacin)*****, c** ***(Chloramphenicol)*****, d (*** ***Tetracycline*** **) from lower to higher concentration (100µg/ml to 10 mg/ml), the culture showed the growth in presence of antibiotics till 1 mg/ml concentration but at 10mg/ml the antibiotic was sensitive.**

Table 5: **MDR test for W1**

Concentration	Amoxycillin	Ofloxacin	Ampicillin	Tetracycline	Ciprofloxacin	Chloramphenicol
10µg	R	R	R	R	R	R
20µg	R	R	R	R	R	R
50µg	R	R	R	S	R	R
100µg	R	R	R	S	R	R
1mg	S	S	S	S	R	S
10mg	S	S	S	S	S	S

R= Resistance, S= Sensitive

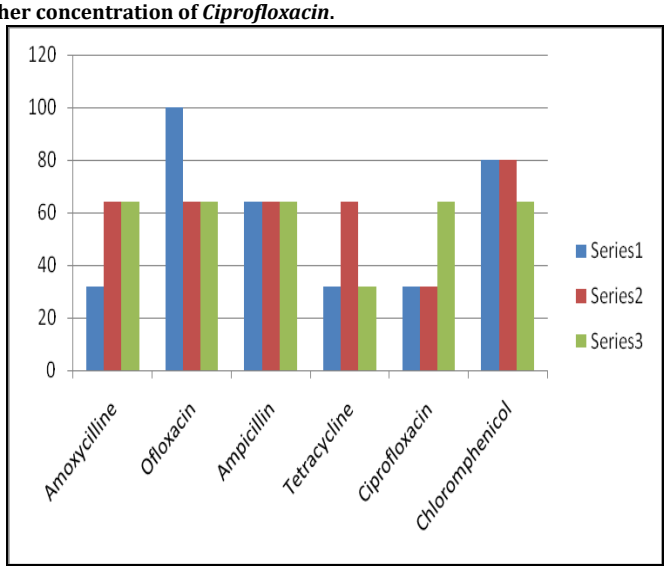
Table 5 **showed that the 6 antibiotics were used** ***Amoxicillin, Ofloxacin, Ampicillin, Tetracycline Ciprofloxacin*** **and** ***Chloramphenicol*** **and after getting result it was clear that the culture W1 was showing resistant at higher concentration of** ***Ciprofloxacin*****.**

Table 6: **% Activity of resistance for all cultures against antibiotics**

Antibiotics	% activity of Culture D2	% activity of Culture S2	% activity of Culture W1
Amoxycillin	32	64	64
Ofloxacin	100	64	64
Ampicillin	64	64	64
Tetracycline	32	64	32
Ciprofloxacin	32	32	64
Chloramphenicol	80	80	64

Table 6 **showed that for all antibiotics, all the cultures were showing resistance and against Ofloxacin it was 100%.**

Graph1: % activity of resistance of all the cultures against all the antibiotics



Series1 =D2 culture, series 2= S2 culture and Series 3= W1 culture

Graph 1 **showed that the maximum resistance was obtained for culture D2 for ofloxacin.**

MIC: (Minimum Inhibitory Concentration)

Table 6: **MIC of sample D2 and S2 culture**

Test tubes	Concentration (mg/ml)	Tetracycline for S_2 culture (O.D at 600 nm)	Ofloxacin for D_2 culture (O.D at 600nm)
1	0.062	0.04	0.01
2	**0.015**	0.10	**0.00**
3	**0.00375**	**0.00**	0.01
4	0.00098	0.01	0.01
5	0.00024	0.01	0.36
6	0.00006	0.10	0.37

Table 6 **showed that the least concentration was obtained 0.015 mg/ml for Ofloxacin against D2 culture and 0.0037 mg/ml for Tetracycline against S2 culture.**

Table 7: **MIC of sample W1 and S2 culture**

Test tubes	Concentration (mg/ml)	Ciprofloxacin for W1 culture (O.D at 600 nm)	Amoxycillin for S_2 culture (O.D at 600 nm)
1	**0.062**	**0.02**	0.01
2	**0.015**	0.04	**0.00**
3	0.00375	0.22	0.28
4	0.00098	0.30	0.37
5	0.00024	0.46	0.36
6	0.00006	0.46	0.20

Table 7 **showed that the least concentration was obtained 0.062mg/ml for Ciprofloxacin against W1 culture and 0.015 mg/ml for Amoxycillin against S2 culture.**

MBC: (Minimum Bacteriocidal Concentration)

Figure 12: **MBC plates of the S2 isolate** **Figure 13: MBC plates of the D2 isolates**

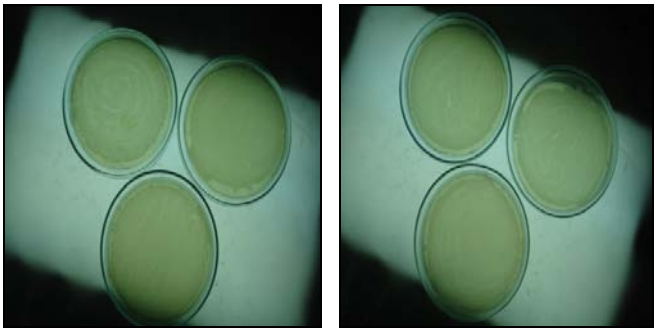


Figure 12 & 13 showed the growth in the presence of antibiotics (Tetracycline, Ofloxacin).

Discussion

Present time there are so many drugs which are used for curing chronic diseases but some cultures are resistant for some antibiotics which are known as multi drug resistant. In this present study there were total 16 culture isolated and out 16, only 3 cultures were identified and further used for experiment. The multi drug resistance property of culture was determined by agar well diffusion method [11] and the cultures *S. aureus* (W1), *Bacillus* (D2) and *Pseudomonas* (S2) were showing positive result [12]. The results obtained in the form of full growth of culture on a petriplates at higher conc. of antibiotics. The 6 antibiotics were used *Amoxicillin, Ampicillin, Tetracycline, Ofloxacin, Chloramphenicol, Ciprofloxacin* at lower to higher conc. (10µg- 10mg), [13].The best results obtained for *Ofloxacin, Ampicillin, Chloramphenicol* antibiotics (10µg-1mg). The % resistance activity of all the cultures against all the antibiotics showed that the activity was found 100% for Ofloxacin for D2 culture and 80% for Chloramphenicol for D2 culture which was identified as a *Bacillus* culture. The other higher activity was found 80% for Chloramphenicol for S2 culture and for W1 the highest activity was found to be 64 % for almost all the antibiotics. The least concentration was obtained 0.015 mg/ml for Ofloxacin against D2 culture and 0.0037 mg/ml for Tetracycline against S2 culture and 0.062mg/ml for Ciprofloxacin against W1 culture and 0.015 mg/ml for Amoxycillin against S2 culture.

Conclusion:

At the end of this experiment it was identified that out of all bacterial cultures isolated from various types of area, three were multi-drug resistance. Isolates D2 & W1 are gram +ve rods and only one isolate S2 is gram –ve rods. Out of three, two were catalase + ve and one was catalase – ve. D2 was identified as *Bacillus*, S2 was *P. aeruginosa* and W1 was *S.aureus.* All these three isolates are multi-drug resistant as they were resistant to various antibiotics used which were of different mechanism of action. After MIC & MBC test it was observed that the isolated cultures were bacteriostatic in nature**.**

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