Comparative Evaluation of Drug Resistance Among Culturable Bacteria isolated from Pharmaceutical Manufacturing Clusters (PMC) in and around Hyderabad

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Executive Summary

The study is undertaken to find out existence selective enrichment of drug resistant bacteria in and around Pharmaceutical Manufacturing Clusters (PMC) of Hyderabad. While undertaking this study soil and water samples were used to isolate culturable bacteria. The soil and water samples were collected from storm water drains/ canals of PMCs and nearby water tanks. The soil samples collected from locations found 5KM upstream (non-industrial) of PMCs and water tanks located 50 KM away from PMCs served as controls. The culturable bacterial load was calculated from all these places and resistance pattern was determined for ten different antibiotics. The findings are summarized to give overview of the study.

Culturable bacteria represented both gram negative and positive bacteria. Drug resistant bacterial Strains were found both in samples collected from PMCs and outside of PMCs. The samples collected from Nallagandla Lake, located up to 50KM away from any known PMCs, have more or equal number of drug resistant strains for certain antibiotics than in samples collected from within PMCs. Resistant pattern found in bacterial strains found at 1 and 5 KM upstream (non-industrial area) of the Jadcherla SEZ, was strikingly similar. There is no clear evidence of selective enrichment of drug resistant bacteria among soil/water samples collected around PMCs. Further studies are in progress to establish the taxonomic identity of drug resistant strains.

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Introduction:

Antibiotics are a class of drugs used to treat diseases associated with mostly bacterial infections. In certain cases, prolonged exposure to the sub-lethal doses of theses antibiotics, trigger stress responses and subsequently help in development of resistance among pathogenic bacteria. A number of multidrug and total drug resistant bacterial strains have been identified among clinical isolates obtained from sources with a history of prolonged and indiscriminate usage of drugs (*Nature Reviews Microbiology* **8**, 836, December 2010).

Hyderabad is an epicentre for several Pharmaceutical Manufacturing Clusters (PMC). They produce drugs to meet the demands of both domestic and international markets. Recently a number of articles have appeared both in scientific journal and daily news papers reporting on existence of drug resistant bacteria in the vicinity of pharmaceutical manufacturing units. Such claims are based on isolation of resistant bacterial strains in soil and water samples collected from the storm water drains of the PMCs. The reports claim existence of antibiotic residues in the environment which, according to the authors, contribute for triggering resistance for most of the known antibiotics (Marathe et al, 2013). These studies have not examined about the existence of resistant bacteria in soil and water samples collected from natural habitants located away from the PMCs to show that the resistant strains are unique to the soil/waters collected from PMCs. Further no systematic analysis is made to claim existence of antibiotic residues in these samples and to identify if the resistant strains are pathogenic in nature. In the present study we have made systematic analysis of

culturable bacterial found in soil/water collected from PMCs and outside of PMCs and provide status report on occurrence of resistance among bacterial population isolated from PMCs and outside PMCs.

Objectives:

1. To collect soil and water samples from storm water drains/canals Pharmaceutical Manufacturing cluster units
2. To isolate and enumerate drug resistant bacteria from the samples
3. To determine occurrence of multi drug resistance among the isolated bacteria
4. To evaluate incidence of drug resistance among strains isolated from soils collected from native habitats and storm water drains/ canals of PMC units.
5. To establish the taxonomic identity of the multi drug resistant strains.

Methodology:

Sample collection:

Both soil and water samples were collected from the below mentioned areas. Water and soil samples were collected from storm water drains/canals of Isnapur-Pashamylaram Industrial Development Area (IDA)located in the vicinities of Virchow-Gaddapotharam IDA, Ramky- Kazipally IDA, Hetero-Kazipally IDA, seepage - Gaddapotharam IDA, Aurobindo unit VII- Jadcherla SEZ. Water / soil samples collected from Nallagandla Lake located near University of Hyderabad and Jadcherla (rural) located 1 Km and 2 Km away from PMC units were used as controls. In order to have statistically significant results on occurrence of drug resistance, water and soil samples were collected from three different spots of each location and all of them were used to isolate culturable bacteria.

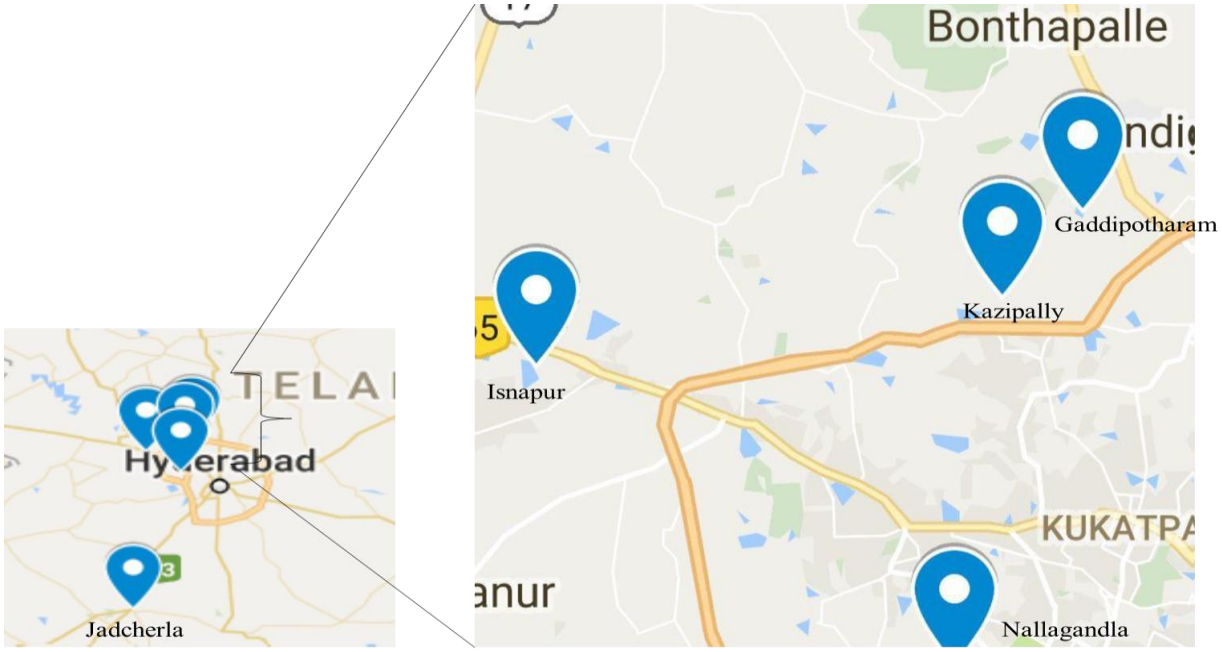

Fig.1: Representative Map indicating the locations at which water and soil samples were collected.

Table. 1 Sample collection Units

Place	Water samples	Soil Samples
Isnapur Lake	I	I
	II	II
	III	III
Gaddapotharam (Near Virchow)	I	I
	II	II
	III	III
Kazipally (Near Ramky)	I	I
	II	II
	III	III
Kazipally (NearHetero)	I	I
	II	II
	III	III
Gaddapotharam (Seepage)	I	I
	II	II
	III	III
Jadcherla (Aurobindo)	I	I
	II	II
	III	III
	IV	-
Jadcherla Rural	-	I (1 Km away from PMC Units)
	-	II (2 km away from PMCUnits)
Nallagandla Lake	I	-

A pictorial representation of sample collection spots is shown in Figure. 1. The samples thus collected were flash frozen and maintained at -30ºC until further use. A detailed table is provided (Table-1) to indicate details of sample collection locations.

Serial dilution of water samples:

The water samples collected from different spots were carefully taken into a clean eppendorf tube and were serially diluted with sterile milliQ water till the sample dilution is reached to 10^{-10}. An aliquot of (100 µl) sample was taken from each dilution and plated on LB plates. The plates were then incubated at 30°C till the bacterial colonies appeared on the plate. In any case the plates were not incubated for more than 24 hours. The dilutions that gave good number of independent colonies were taken for further experiments.

Isolation of bacteria from soil samples:

A gram quantity of soil collected from various collection spots was taken into a clean sterile tube and suspended in 1 ml of sterile water. The contents were stirred vigorously and left on work bench till the suspended solid particles got settled at the bottom of the tube. The clear supernatant was taken into a sterile tube and considered as initial stock.

Isolation of bacterial colonies:

LB-agar plates prepared following standard procedures were used to spread 100µl of sample taken from each dilution. These plates were then incubated at 30ºC until uniformly visible individual colonies were obtained. The plate that gave optimal number of colonies with sufficient inter colony space was taken for further studies.

Colony counting:

The number of the colonies found on each plate were counted and this number was multiplied with the dilution factor to get number of colonies found in one millilitre of sample. The number of colonies found in water and soil samples were reported as X number of colonies/ml in case of water samples and X number of colonies/g of soil in case of soil samples).

Determination of antibiotic resistance:

LB plate which is divided into a number of squares was taken and the colonies that appeared in appropriate dilution were spotted in each square. This plate was incubated for 12 hours to get proper growth of the spotted colony and is used as a master plate while replica plating on LB plate containing Ampicillin (100µg/ml) , Kanamycin (50ug/ml), Tetracycline (20µg/ml), Chloramphenicol (30µg/ml), Cefotaxime (250µg/ml), Cefepime (250µg/ml), Ceftazidime (250µg/ml), Eartapenem (250µg/ml), Cefpodoxime (250µg/ml) and Ciprofloxacin (250µg/ml). The colonies grown on antibiotic containing plate were regrown under similar conditions. If the colony is grown on antibiotic containing plate for three successive generations, then the colony is regarded as antibiotic resistant colony (Fig.3).

Identification of Multi drug resistant strains:

The colonies which were resistant to 3 or more antibiotics were termed as multi drug resistant strains and such colonies were selected for determining taxonomic position by determining nucleotide sequence of 16S rRNA coding gene.

Amplification of 16S rRNA coding gene:

The resistant colonies were taken with a sterile tooth pick and resuspended in 20µl of sterile water. All these tubes were then kept in 98ºC bath for 10 minutes

to ensure complete lysis of the cell. After lysis the contents were collected to the bottom of the tube by brief centrifugation and stored at 4ºC until further use. When necessary 2 µl of the sample was taken to perform PCR to amplify 16s rRNA coding gene using Emerald Master Mix (Takara). The sequences of the primers used in this study are mentioned below.

8F: AGA GTT TGA TCC TGG CTC AG

U1492R: GGT TAC CTT GTT ACG ACT T

After PCR a portion of the reaction mix was analyzed on the 0.8% agarose gel to check the amplification. If the amplicon size is matched with the size of 16S rRNA coding gene (1. 5 Kb), the remaining portion of PCR mix was used to purify the amplicon and to determine its nucleotide sequence. The agarose gel showing the amplification of 16S rRNA coding gene is shown in Fig. 2

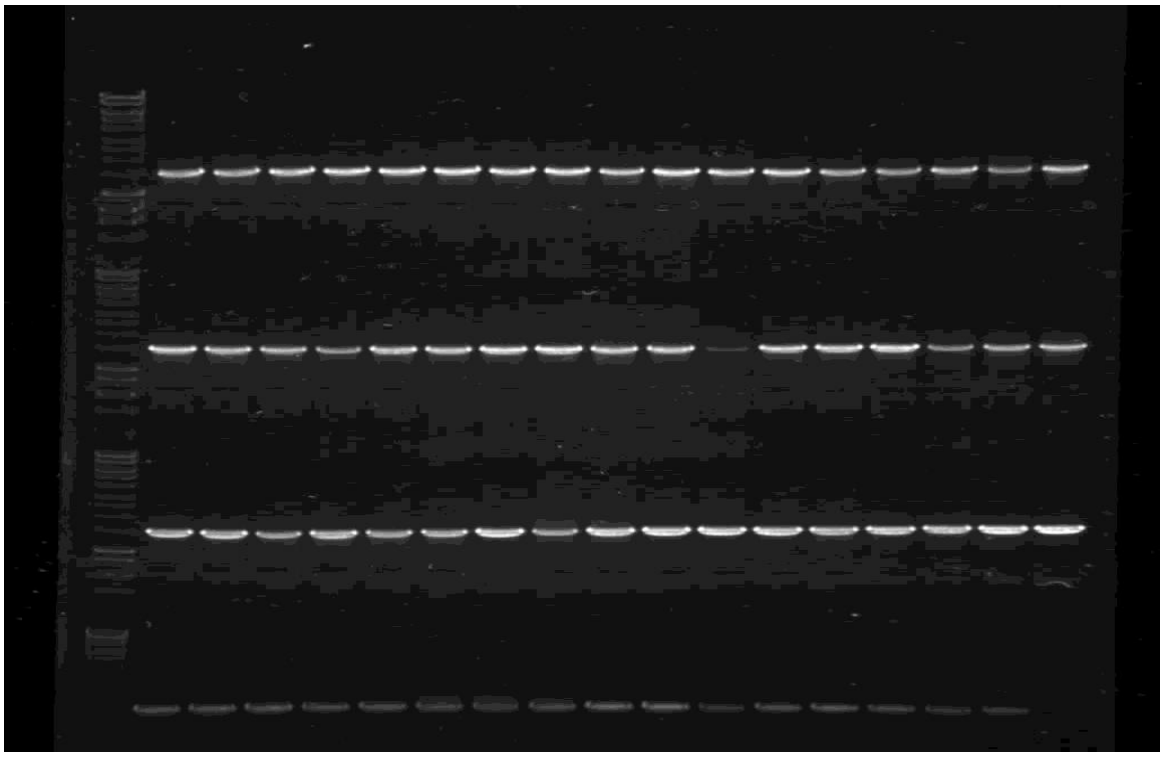

Fig.2: Representative image of agarose gel showing amplification of 1.5 kbp 16S rRNA coding gene.

Sequencing and determination of phylogenetic tree:

The 16S rRNA gene was sequenced by sending PCR products to M/S. Regene Biologicals Pvt Ltd. Hyderabad. The sequencing was determined using aforementioned primers. After obtaining the sequences, the sequence was edited to exclude the PCR primer binding sites and to make corrections if any. The complete 16s rRNA gene sequence obtained was used to perform BLAST similarity with known sequences (http://www.ncbi.nlm.nih.gov/). The phylogenetic analysis will be constructed using MEGA version 6.0 software.

Results:

Bacterial load in collected soil and water samples:

Before proceeding to identify drug resistant bacteria in each of the collected samples, the total bacterial count was determined by following method described in materials and methods section. The number of the colonies found at each of the collected soil and water samples were given in the table-2 and 3 respectively. As soil and water samples were collected from three independent spots from each of the locations the average number of colonies obtained from each location was used to spot the graph.

Determination of drug resistance among culturable bacteria:

The figure shown below describes identification process by which drug resistant strains were identified from among the total bacterial population. Initially master plate having clearly developed colonies with sufficient inter colony space was taken and each colony was plated on LB plate divided into clearly numbered squares. Similar plates were prepared by adding specified concentrations of antibiotics mentioned in materials and methods. The colonies that showed resistance for three or more antibiotics were taken for taxonomic identification. Samples collected from Nallagandla lake and 1 & 2 Km away

from Jadcherla SEZ, were processed in similar manner and used as control samples.

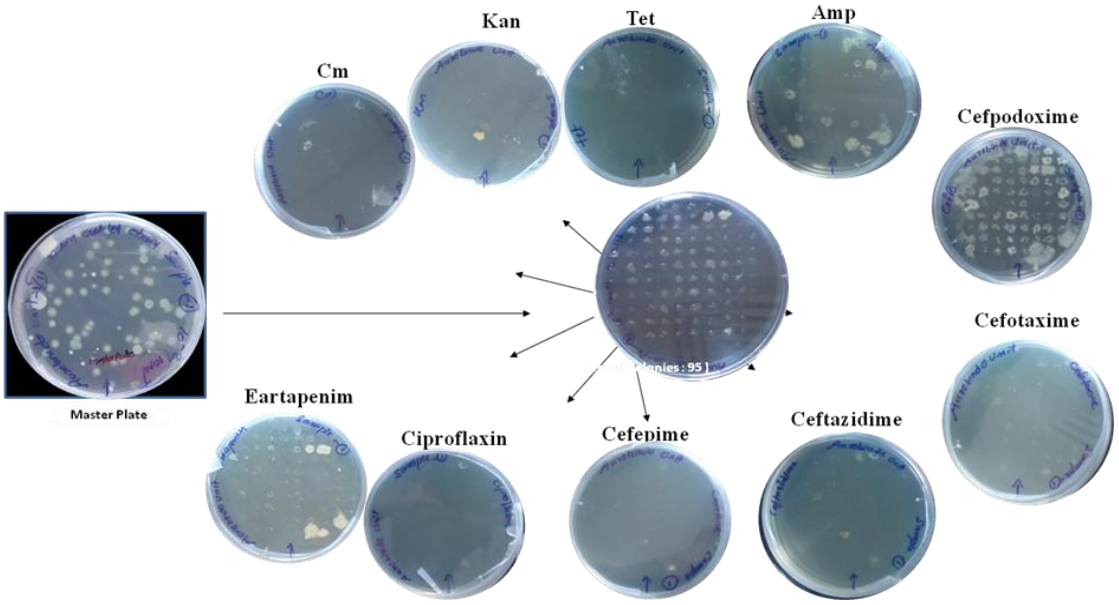

Fig. 3: Representative image of master plate and plates with antibiotics.

Table. 2 Number of colony forming units in Soil samples:

Place	Sample Number	Dilution factor	No of colonies in LB	Total No of colonies per g soil
Isnapur lake	I	10^3	23	230000
	II	10^3	46	460000
	III	10^3	52	520000
Gaddapotharam (Near Virchow)	I	10^3	90	90000
	II	10^2	100	100000
	III	10^2	87	87000
Kazipally (Near Ramky)	I	10^2	60	60000
	II	10^2	48	48000
	III	10^2	50	50000
Kazipally (Near Hetero)	I	10^2	66	66000
	II	10^2	100	100000
	III	10^2	102	102000
Gaddapotharam (Seepage)	I	10^1	69	6900
	II	10^1	62	6200
	III	10^1	75	7500
Jacherla-SEZ (Aurobindo)	I	10^2	50	50000
	II	10^2	50	50000
	III	10^2	100	100000
Jadcherla Rural	I (1 Km)	10^1	160	16000
	II (2 km)	$10^{0.5}$	120	6000

Table. 3 Number of colony forming units in water samples:

Place	Sample Number	Dilution factor	No of colonies in LB	Total No of colonies per mL
Isnapur lake	I	10^3	31	310000
	II	10^3	68	680000
	III	10^1	234	234000
Gaddaptharam (Near Virchow)	I	10^1	169	16900
	II	10^1	203	20300
	III	10^1	223	22300
Kazipally (Near Ramky)	I	10^1	60	6000
	II	10^1	40	4000
	III	10^1	65	6500
Kazipally (Near Hetero)	I	10^1	58	5800
	II	10^1	80	8000
	III	10^1	58	5800
Gaddapotharam (Seepage)	I	10^4	68	680000
	II	10^2	84	84000
	III	10^1	38	3800
Jadcherla, SEZ (Aurobindo)	I	10^1	150	15000
	II	10^1	180	18000
	III	10^1	258	25800
	IV	10^1	190	19000
Nallagandla lake	I	10^1	70	7000

1) Culturable bacterial load obtained in Soil samples:

The antibiotic residues if mixed with water will be adsorbed to soil particles and get percolated into soil and ground water. Such condition will be highly reflective of sublethal exposure of antibiotic residues to soil bacteria. If grown for several generations there is ample scope for development of resistance to antibiotics. Therefore we have collected soil samples from PMC's (bulk drug manufacturing units) and from locations which have no laboratory of antibiotics producing units. The soil samples were analysed to know the colony forming units and to evaluate drug resistant colonies from isolated bacterial load. The total number of cfu's obtained from each location is shown in table-2. The average of all antibiotic resistant strains obtained in a particular location was plotted by taking percent resistance on y-axis and antibiotics on x-axis.

Isnapur:

A total of 2,30,000 colonies were obtained per gram of soil collected next to lake (sample-1). Similarly the culturable bacterial load in sample -2 was 4,60,000 colonies per gram of soil. The bacterial load was double than the bacterial load obtained in sample -1. In sample -3, 5,20,000 colony forming units were obtained per gram soil (Fig.1). This load is significantly higher than the load obtained in sample -1 but lesser than the load obtained in sample -2. The number of cfu per gram of soil, primarily depends on the nature of the soil and its fertility. In order to gain consistency on drug resistant colonies, the average of cfu's resistant to each antibiotic was calculated and graph was plotted to indicate percent resistance. On an average, the bacterial load obtained per gram soil in this location was found to be 4,03,333 of which 30.7% were resistant to Ampicillin, 24.8% to Kanamycin, 3.7% to Tetracycline, 29.2% to Chloramphenicol, 23.4% to Cefotaxime, 7.2% to Cefepime, 20% to Ceftazidime, 0% to Eartapenem, 47.2% to Cefpodoxime and 1.4% to ciprofloxacin. The highest resistance is seen towards cefpodoxime and lowest to ciprofloxacin. Interestingly there was no resistance to eartapenem (Fig.4A).

The resistance obtained at this location was then compared with that of the data generated from control soil samples collected from Nallagandla Lake and Jadcherla village. As shown in Fig. 10B more resistant colonies were found for cefepime in bacteria isolated from Nallagandla lake and Jadcherla village. However for cefpodoxime more resistant colonies were found at Jadcherla village. Unlike the resistance observed in water samples, resistance to cefepime are more in cfu's obtained in soil samples collected from Nallagandla lake. However the resistance with most of the antibiotics remain unaltered (4B).

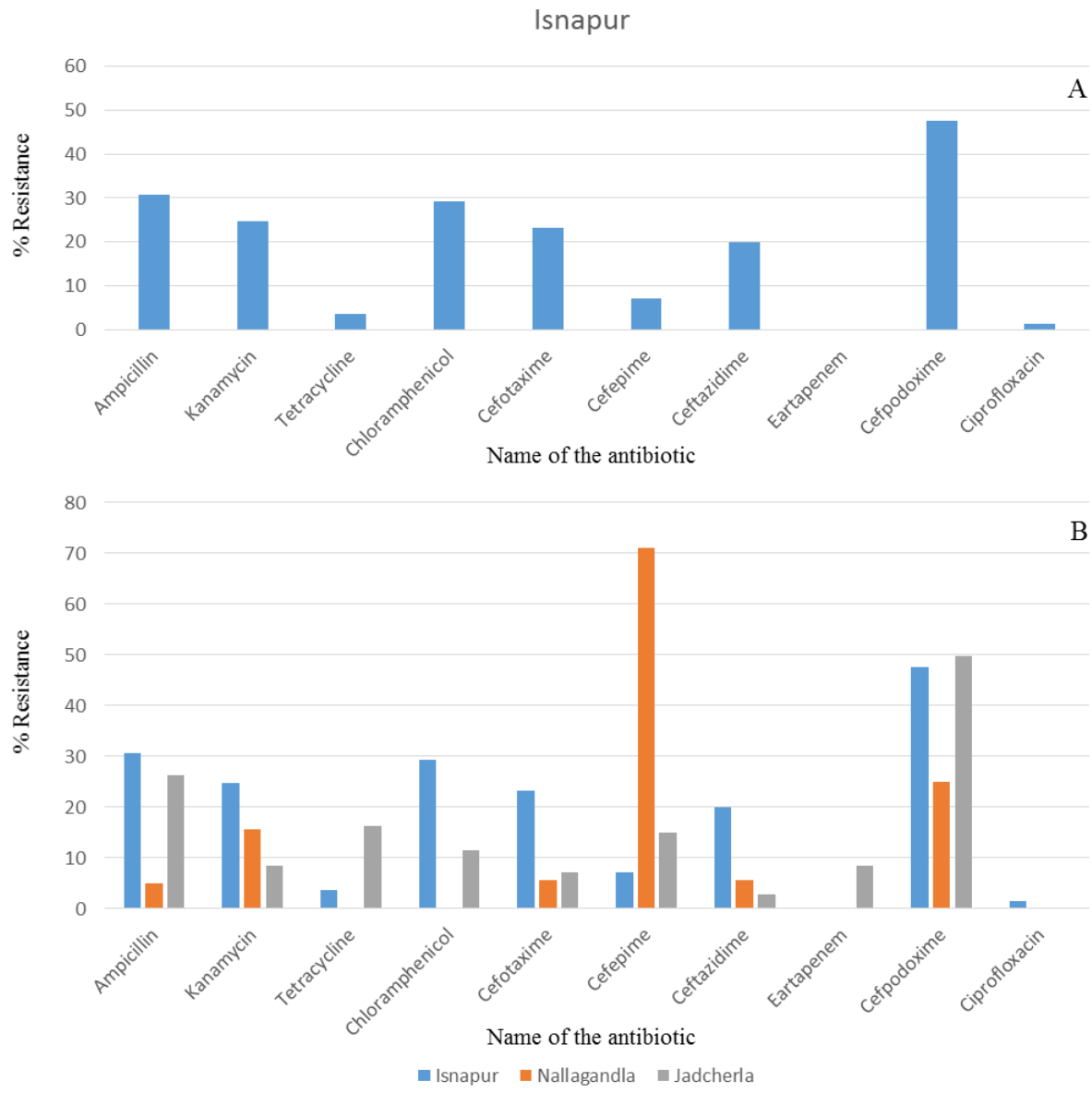

Fig. 4: Drug resistance pattern among cfu's isolated from soil samples collected at Isnapur (A), Jadcherla village and Nallagandla lake taken as control (B).

Gaddapotharam (Near Virchow):

A total of 90,000, 100,000 and 87,000 colony forming units were obtained per gram of soil collected from Gaddapotharam at spots 1, 2 and 3 (Fig.1) respectively. Spot -2 gave highest bacterial load compared to the other 2 spots.

The data obtained was analyzed and a graph was plotted against the average resistance obtained towards each antibiotic.

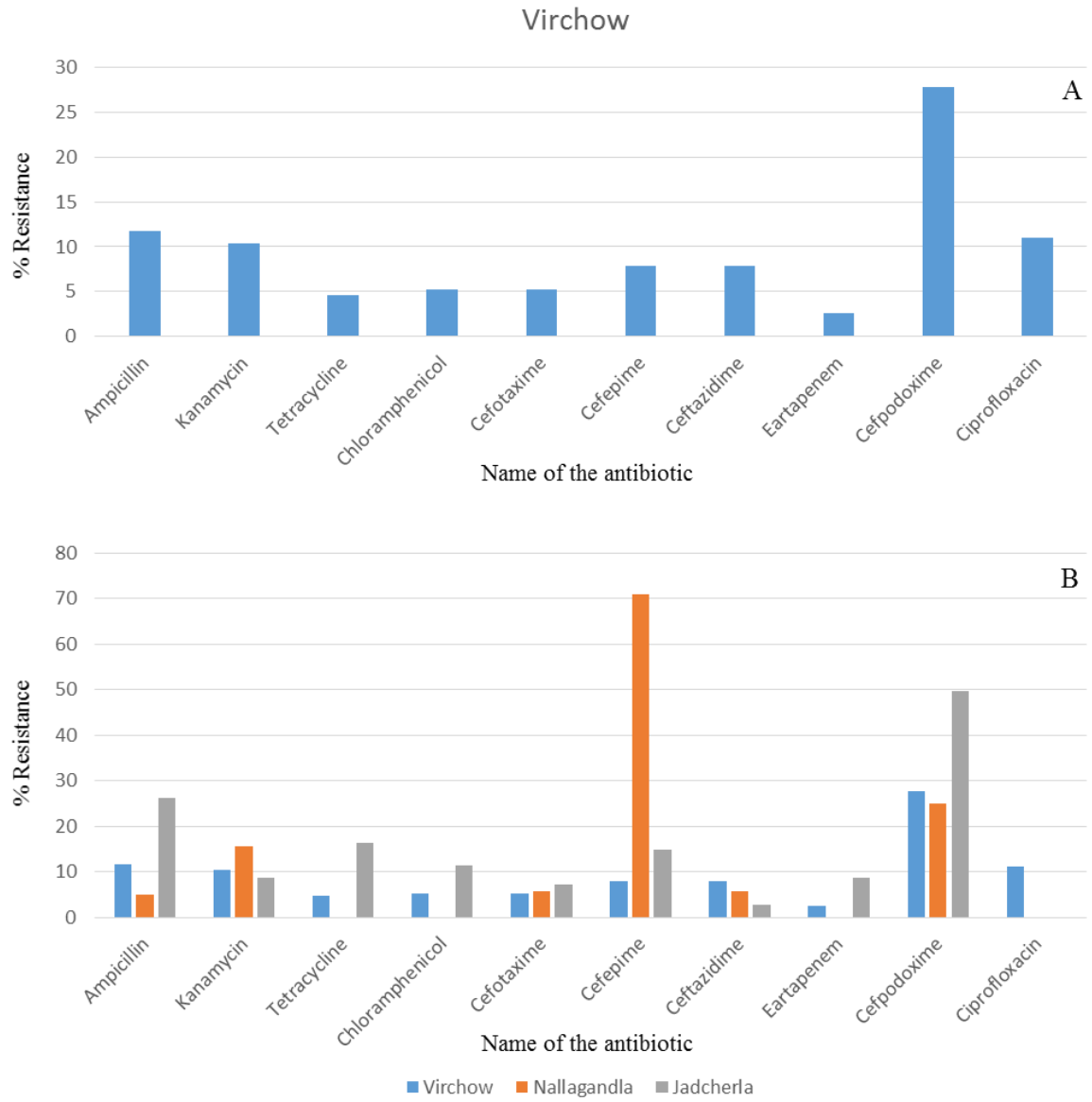

Fig. 5: Comparison of drug resistance pattern among cfu's isolated from soil samples collected near Virchow labs (A), Jadcherla village and Nallagandla lake taken as control (B).

The average bacterial load per gram soil was found to be 92,333 cfu's out of which 11.7% were resistant to Ampicillin, 10.4% to Kanamycin, 4.7% to Tetracycline, 5.2% to Chloramphenicol, 15.6% to Cefotaxime, 7.8% to Cefepime, 7.9% to Ceftazidime, 2.6% to Eartapenem, 27.8% to Cefpodoxime and 11% to Ciprofloxacin. The highest resistance is seen towards Cefpodoxime and lowest to eartapenem. Interestingly there was no resistance to eartapenem (Fig.5A). The resistance obtained at this location was then compared with that

of the data generated from control soil samples collected from Nallagandla lake and Jadcherla village. As shown in Fig. 5B more resistant colonies were found for cefepime in bacteria isolated from Nallagandla lake and Jadcherla village. However for Ampicillin, Tetracycline and Cefpodoxime more resistant colonies were found at Jadcherla village (5B). Interestingly both of them were taken as control samples.

Kazipally (Near Ramky):

The culturable bacterial load obtained per gram of the soil collected from at Spot -1 was 60,000. Similarly at spot -2 and 3 the culturable bacterial load obtained per gram soil was 48,000 and 50,000 cfu's respectively (Fig. 1).

The bacterial load obtained at spot 1 is significantly higher than the other two spots. The bacterial load obtained at spots 2 and 3 were almost similar. The data of all the strains isolated from the 3 spots were analyzed and the average bacterial load obtained at this location was found to be 52,666 colonies per gram soil collected at this location. Out of which 28% were resistant to Ampicillin, 20.8% to Kanamycin, 17.5% to Tetracycline, 12.8% to Chloramphenicol, 40.2% to Cefotaxime, 16.8% to Cefepime, 17.5% to Ceftazidime, 39.3% to Eartapenem, 17.4% to Cefpodoxime and 5.2% to Ciprofloxacin. The highest resistance is seen towards cefotaxime and lowest to Ciprofloxacin (Fig.6A). The resistance towards the antibiotics used in this study obtained at this location was then compared with that of the data generated from control soil samples collected from Nallagandla lake and Jadcherla village. More resistant colonies were found for cefepime and cefpodoxime in bacteria isolated from both Nallagandla lake and Jadcherla village. However for Ampicillin, Kanamycin, Tetracycline, Chloramphenicol, Cefotaxime, Ceftazidime and eartapenem more resistant colonies were found in soils collected from Ramky labs (6B).

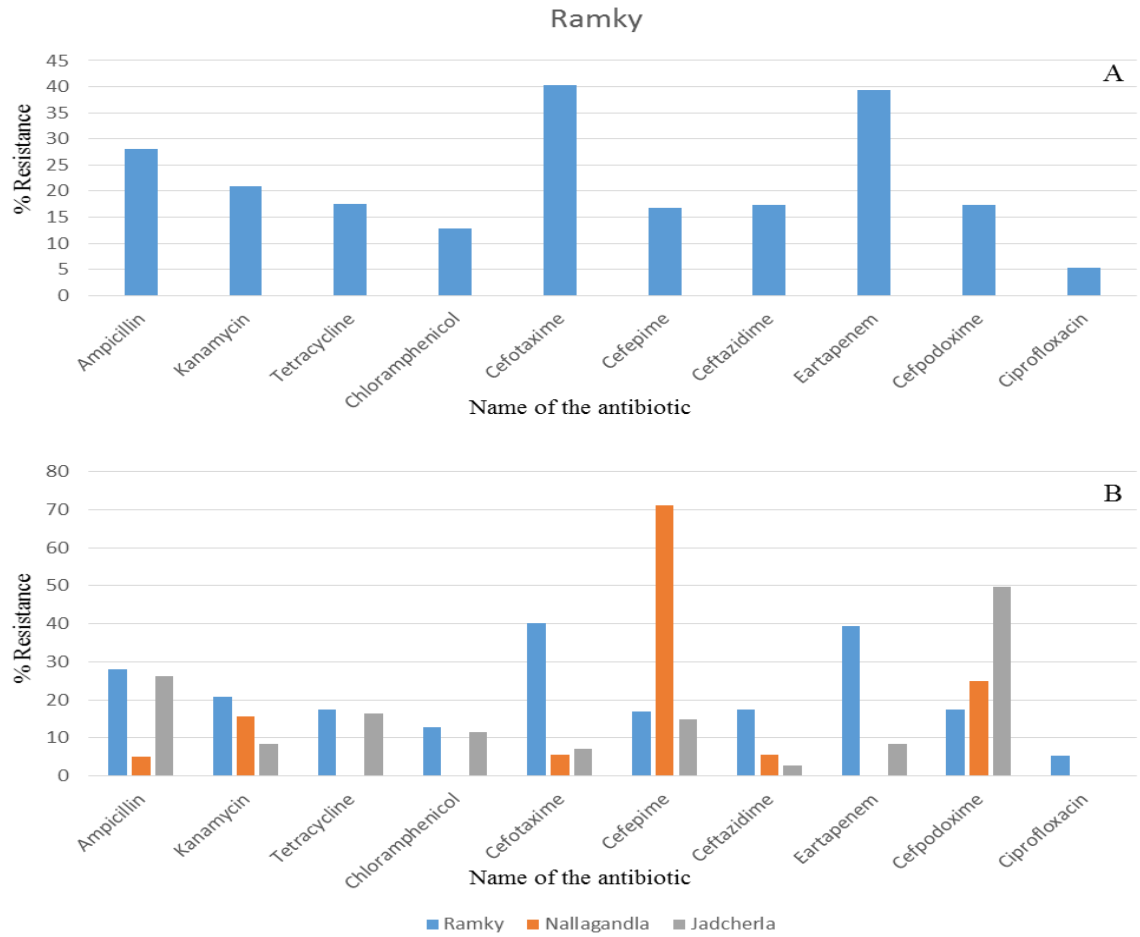

Fig. 6: Comparison of drug resistance pattern among cfu's isolated from soil samples collected near Ramky (A), Jadcherla village and Nallagandla lake taken as control (B).

Kazipally (Near Hetero):

The number of colony forming units obtained per gram soil collected from sample-1 were 66,000. Similarly 1,00,000 and 1,02,000 Cfu's were obtained per gram soil collected from samples 2 and 3 respectively (Fig.1). The culturable bacterial load isolated from spot- 1 is significantly lower than the bacterial load obtained at spots 2 and 3. However no significant difference was observed between bacterial load obtained at spots 2 and 3. The data of all the cfu's isolated from the 3 spots were analyzed and the average bacterial load obtained at this location was found to be 89,333 colonies per gram soil out of which 20.7% were resistant to Ampicillin, 31.1% to Kanamycin, 8.8% to Tetracycline,

18% to Chloramphenicol, 6.6% to Cefotaxime, 12.9% to Cefepime, 9.9% to Ceftazidime, 8.8% to Eartapenem, 84.3% to Cefpodoxime and 8% to Ciprofloxacin. The highest resistance is seen towards cefotaxime and lowest to ciprofloxacin (Fig.7A). The resistance towards the antibiotics used in this study obtained at this location was then compared with that of the data generated from control soil samples collected from Nallagandla lake and Jadcherla village as shown in Fig. 13B. More resistant colonies were found for Cefepime in bacteria isolated from both Nallagandla lake and Jadcherla village. The cfu's collected from soil at Ramky labs showed more resistance to Kanamycin, Chloramphenicol, Ceftazidime and Ciprofloxacin (7B).

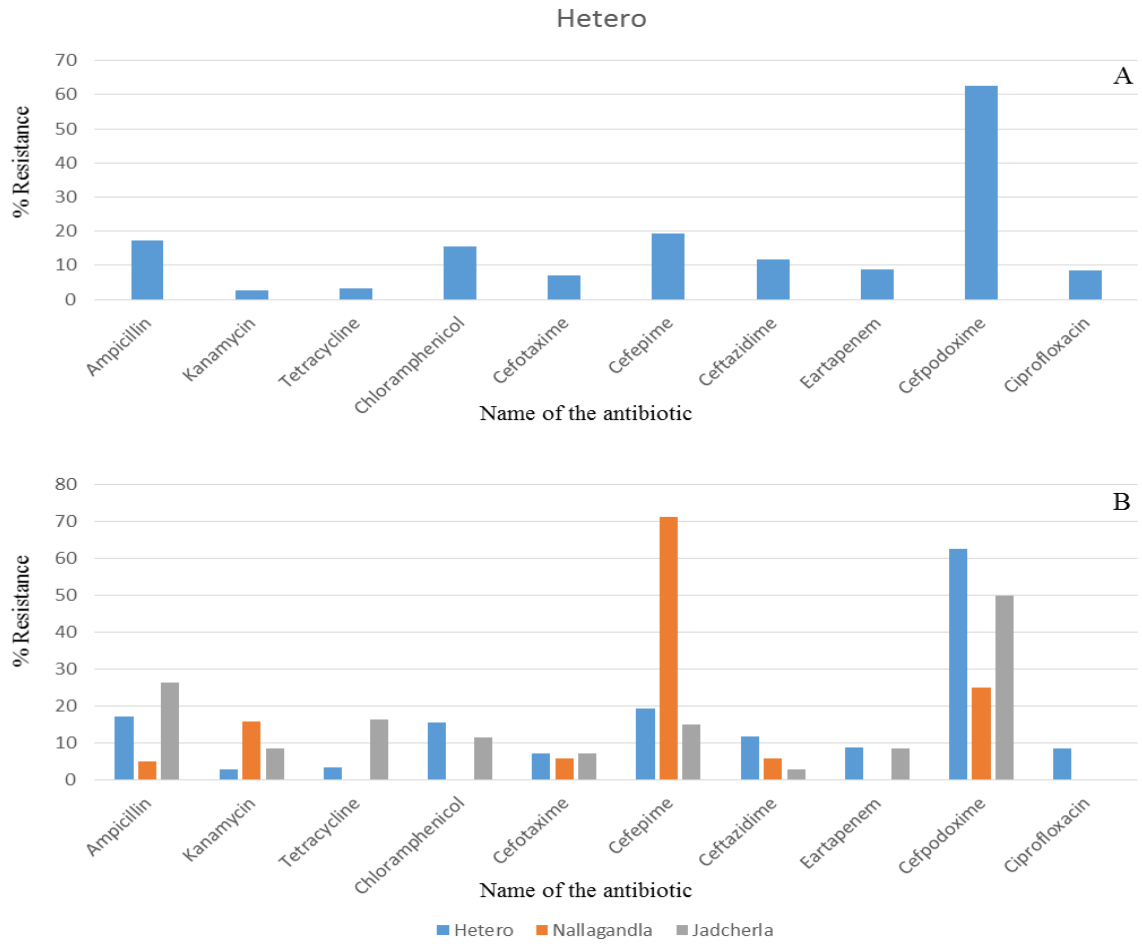

Fig. 7: Comparison of drug resistance pattern among cfu's isolated from soil samples collected near Hetero (A), Jadcherla village and Nallagandla lake taken as control (B).

Gaddapotharam (Seepage):

The number of colony forming units obtained per gram soil collected from spot-1 (In the Circular tank) was found to be 6,900 per gram soil. Similarly, 6,200 and 7,500 cfu's were obtained from soils collected at spots 2 and 3 respectively. The average bacterial load obtained in this location was found to be 6,850 cfu's per grams soil. 17.7% were resistant to Ampicillin, 2.7% to Kanamycin, 3.3% to Tetracycline, 15.6% to Chloramphenicol, 7.2% to cefotaxime, 19.2% to cefepime, 11.6% to ceftazidime, 8.8% to Eartapenem, 62.5% to Cefpodoxime and 8.4% to Ciprofloxacin. The highest resistance is seen towards cefpodoxime and lowest to Kanamycin (Fig.8A).

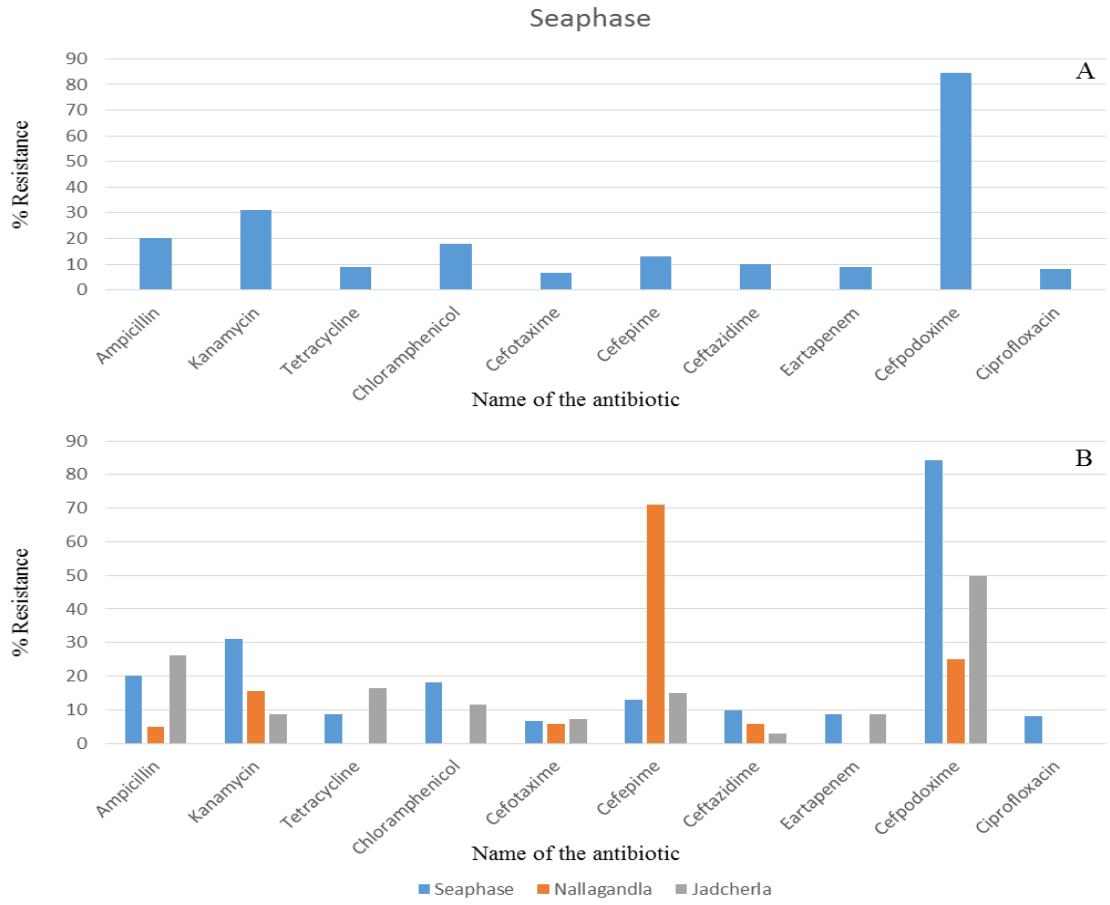

Fig. 8: Comparison of drug resistance pattern among cfu's isolated from soil samples collected at Seepage (A), Jadcherla village and Nallagandla lake taken as control (B).

The resistance towards Kanamycin, Tetracycline, Cefepime, Ceftazidime, and Cefpodoxime in control samples is higher than the soil collected from this location whereas resistance to Chloramphenicol, Ceftazidime and Ciprofloxacin is higher than the bacterial resistance obtained at Nallagandla lake and Jadcherla village (Fig.8B).

Jadcherla, SEZ (Aurobindo)

The number of colony forming units obtained per gram soil collected near outlet drain (Sample-1) was 50,000 colonies. Similarly 50,000 and 100,000 colonies were obtained from soil samples collected at spots 2 (line near road) and 3 (inside the unit) respectively. The bacterial load at spot -3 is double than the load obtained at spots 1 and 2.

The bacterial load and resistance to antibiotics at all the spots in this location were analyzed and the average of all antibiotic resistant strains were plotted by taking percent resistance on y-axis and antibiotics on x-axis. The average bacterial load obtained in this location was found to be 66,666 cfu's per grams soil of which 44.7% were resistant to Ampicillin, 49.5% to Kanamycin, 5.6% to Tetracycline, 43% to Chloramphenicol, 33.3% to Cefotaxime, 37.7% to Cefepime, 37% to Ceftazidime, 15.4% to Eartapenem, 64.6% to Cefpodoxime and 18.9% to Ciprofloxacin. The highest resistance is seen towards cefpodoxime and lowest to tetracycline (Fig.9A).The data of all the cfu's isolated from the 3 spots at this were analysed and compared to antibiotic resistance obtained from soils samples collected at Nallagandla lake and Jadcherla village. As shown in Fig. 9B resistance to Ampicillin, Kanamycin, Chloramphenicol, Cefotaxime, Ceftazedime, Eartapenem and Ciprofloxacin was more in cfu's obtained at this location when compared with the cfu's obtained at control sites. However more resistance towards Tetracycline, Cefepime and Cefpodoxime were shown by bacterial load obtained at control location. (Fig. 9B).

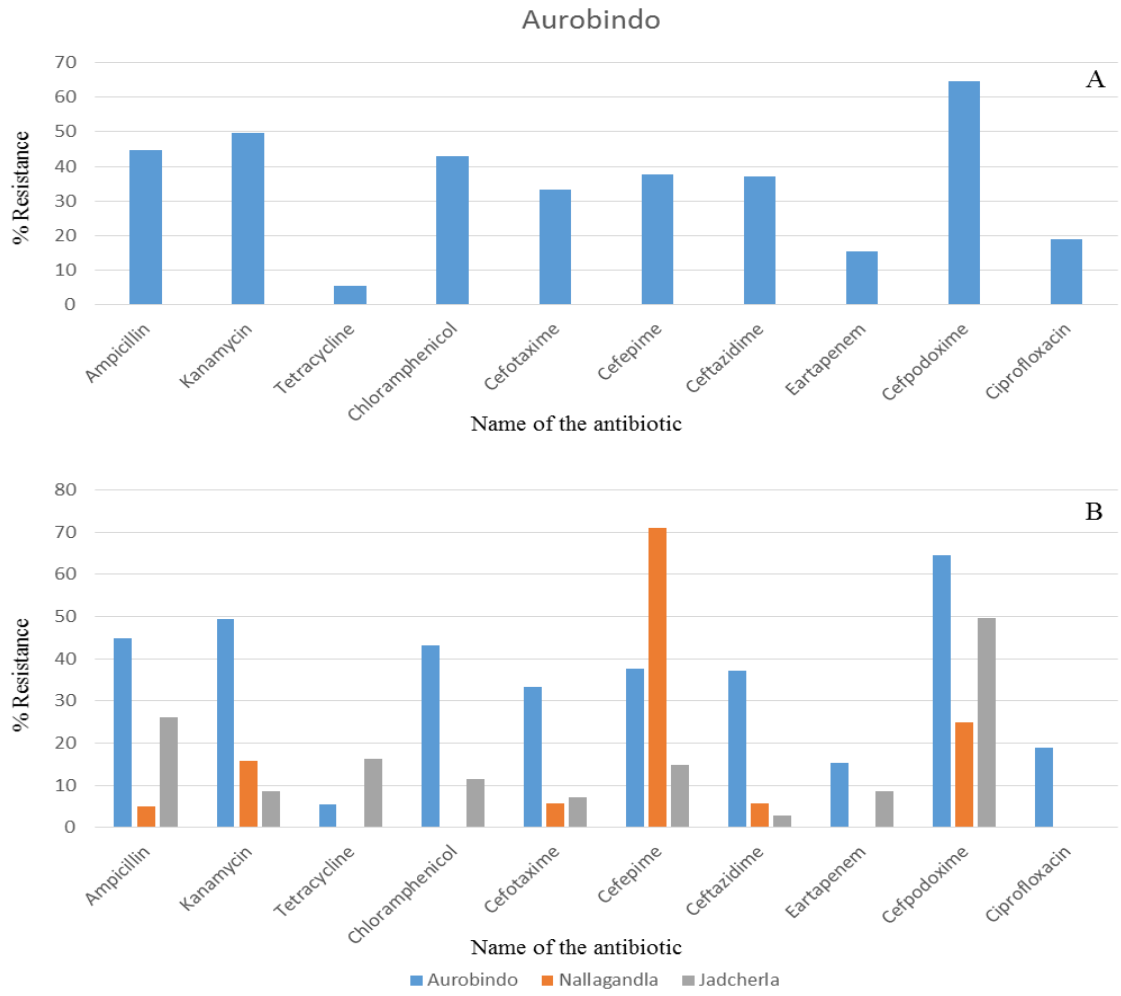

Fig. 9: Comparison of drug resistance pattern among cfu's isolated from soil samples collected at Aurobindo unit VII (A), Jadcherla-SEZ village and Nallagandla lake taken as control (B).

2) Culturable bacterial load obtained in water samples:

Isnapur:

As mentioned in materials and methods, water samples were collected from three independent spots of Isnapur lake to get better information on drug resistant bacteria (Fig.1). Sample -1 collected from inside the lake contained about 3,10,000 colony forming units (cfu's) per ml. Similarly in sample – 2 collected from downstream (outside) of the Isnapur Lake, the culturable bacterial load was 6,80,000 obtained per ml. The bacterial load obtained per ml

of water collected from sample 2 appears to be significantly higher. The load is almost double than the load obtained per ml in sample -1. In sample-3 collected 15 meters away outlet from Isnapur lake gave 2,34,000 colonies per ml. The bacterial load obtained at this spot is significantly less compared to the other two spots.This may be due to location of the sample collection spot. The sample collected downstream of the lake means from the overflow of Isnapur lake. In the Lake there will have lot of organic matter that facilitates the growth of the bacteria. Since these bacterial population flow along with water currents, the water collected from outlet will have more number of colony forming units. The data clearly suggest that higher number of bacterial population in the Lake due to accumulation of organic material in the water. In the inlet less number of colony forming units are observed. The cfu's got enriched in the lake. This number is significantly higher in the water outlet (Over flow).

To minimize the error, the total bacterial load was calculated by taking the average of the bacterial loads obtained at all the three spots. The colony forming unit on an average at this location was found to be 4,08,000 per ml. Further the average number of the resistant colonies obtained for each of the 10 antibiotics were taken and a graphical representation was made to represent resistant patterns of each location. We observed that 61% of the colonies were resistant to Ampicillin, 7.5% to Kanamycin, 2.4% to Tetracycline, 3% to Chloramphenicol, 13.4% to Cefotaxime, 2.1% to Cefepime, 9.8% to Ceftazidime, 2.5% to Eartapenem, 38.4% to Cefpodoxime and 1.2% to Ciprofloxacin. As per the generated data, in Isnapur Lake the highest resistivity was observed towards ampicillin whereas the lowest resistance was seen towards ciprofloxacin (Fig.10A).

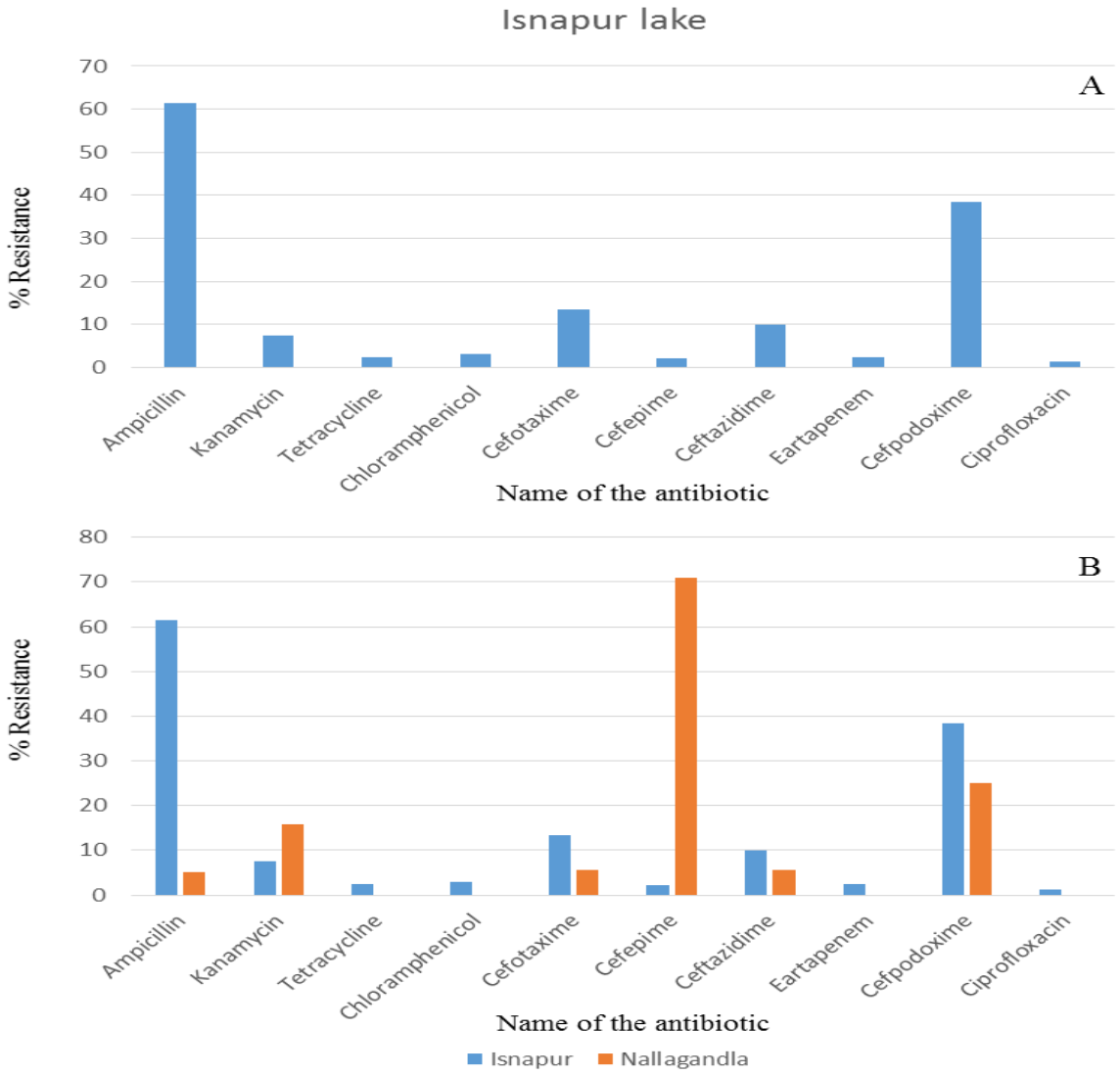

Fig. 10: Comparison of drug resistance pattern among cfu's isolated from Isnapur lake (A) and Nallagandla lake taken as control (B).

Gaddapotharam (Near Virchow):

A total of 16,900, 20,300 and 22,300 colony forming units were obtained per ml in water samples collected from downstream of boundary wall water (sample-1), adjacent to labs (sample-2) and lake located near Virchow labs (sample-3) respectively (Fig.1). All these spots are located near Virchow unit of Gaddapotharam IDA. The total colony forming units obtained at spot 1 are significantly lower than cfu's obtained at spots 2 and 3. There seemed to be less significant difference in cfu's at spots 2 and 3. However the number of CFU at

spot 3 were highest. Again the spot – 3 is within the lake which is loaded with organic matter that supports the growth of bacteria.

An average number of colonies resistant to each of the antibiotic used in this study were taken into consideration to plot a graph takin % on y-axis and antibiotics on the x-axis. We observed that 56% were resistant to Ampicillin, 1.1% to Kanamycin, 0.4% to Tetracycline, 29.5% to Chloramphenicol, 0% to Cefotaxime, 23% to Cefepime, 1.1% to Ceftazidime, 23.5% to Eartapenem, 44.3% to Cefpodoxime and 1.2% to Ciprofloxacin. Interestingly there was no resistance observed towards Cefotaxime (Fig.11A).

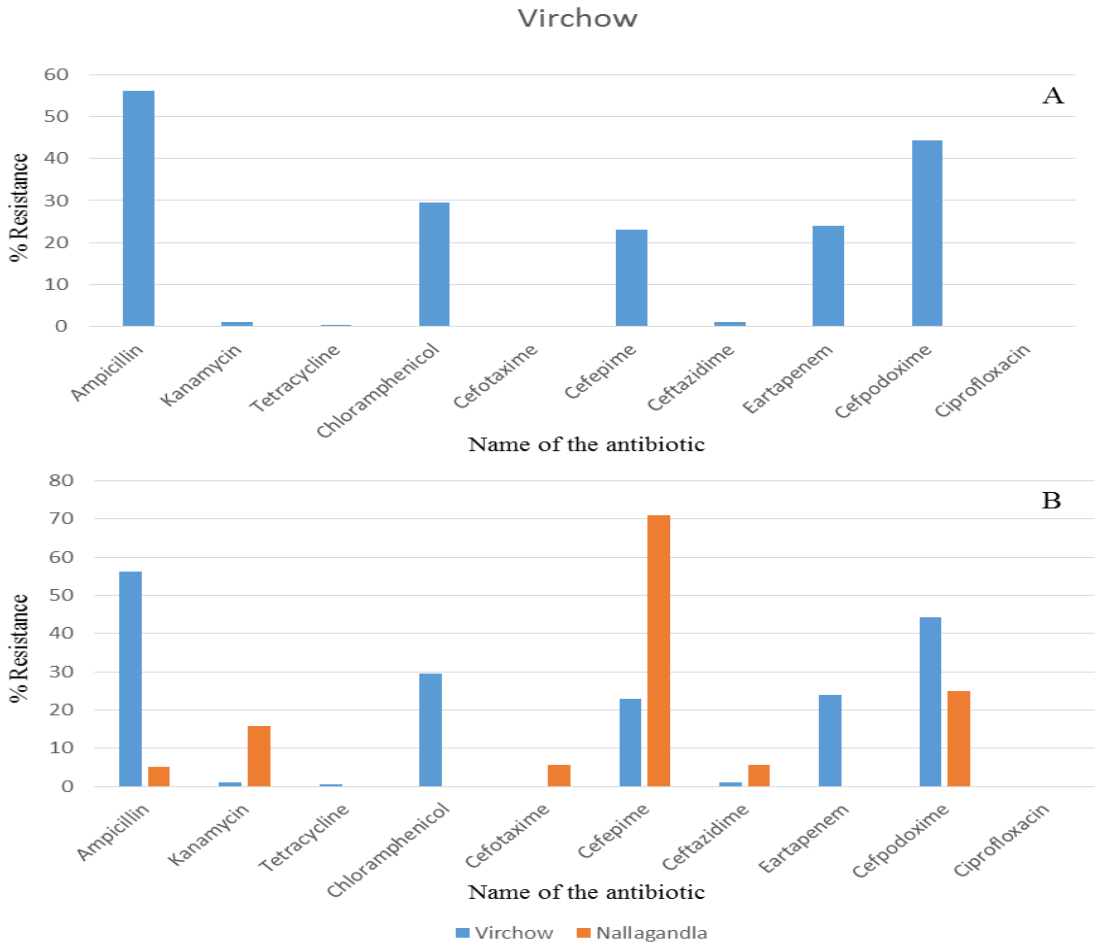

Fig. 11: Comparison of drug resistance pattern among cfu's isolated from water samples collected near Virchow labs-Gaddapotharam (A) and water of Nallagandla lake taken as control (B).

The resistance towards Ampicillin, Chloramphenicol, Eartapenem and Cefpodoxime in water samples collected from Virchow labs appears to be significantly higher than the controls. However the cfu's obtained from Nallagandla lake have shown more resistance to kanamycin, Cefotaxime, Cefepime and Ceftazidime than the counter parts found at Virchow lab water samples. The highest resistivity was observed towards Ampicillin whereas the lowest resistance was observed towards Tetracycline (Fig.11B).

Kazipally (Near Ramky):

A total of 6,000 colony forming units were obtained per ml of water sample collected from water canal found at back side of the unit (Spot-1). The Spot 2 represents canal water located adjacent to kekule pharma and spot 3 represents 100 m away from manufacturing unit (Fig. 1). The number of cfu's obtained per ml were 4,000 and 6,500 respectively. The total bacterial load obtained at spots 1 and 3 were almost similar whereas the bacterial load found at spot-2 was significantly lesser than the other two spots. This may be due to the location of collection spots. It is storm water canal entry pointand hence there will be limited amounts of nutrients which leads to the poor propagation of bacteria.

The data obtained from all the three spots in this location was averaged for each of the antibiotic used and a graph was plotted. It is seen that on an average the culturable bacterial load obtained in this location is 5, 500 per ml. Out of which 7.3% were resistant to Ampicillin, 0.6% to Kanamycin, 0% to Tetracycline, 0% to Chloramphenicol, 15.5% to Cefotaxime, 10.7% to Cefepime, 0% to Cftazidime, 20.7% to Eartapenem, 66.7% to Cefpodoxime and 2.1% to Ciprofloxacin. The highest resistivity was observed towards Cefpodoxime and the lowest resistance was observed towards Kanamycin (Fig.12A). Interestingly no resistance was observed towards Tetracycline, Chloramphenicol and Ceftazidime. As observed in Isnapur and Virchow labs the resistance towards

Ampicillin, Chloramphenicol, Eartapenem and Eefpodoxime in water samples collected from Ramky are significantly higher than the controls whereas Kanamycin, Cefotaxime, Cefepime and Ceftazidime resistance is significantly lower than the bacterial resistance obtained at Nallagandla lake (Fig.12B).

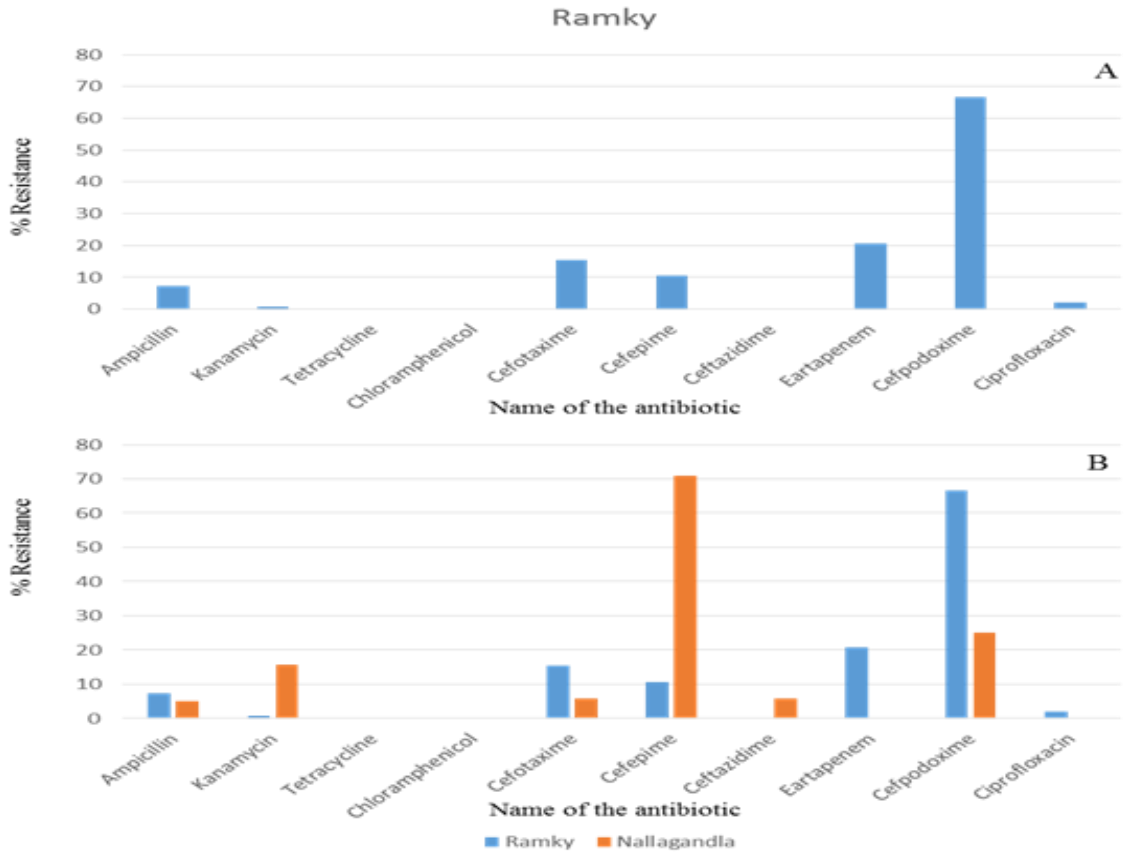

Fig. 12: Comparison and evaluation of drug resistance pattern between water samples collected near Ramky-Kazipally (A) and Nallagandla lake taken as control (B).

Kazipally (Near Hetero):

From units of Hetero located at Kazipally, 3 samples were collected at downstream circular wall (Sample-1), 50 m away to circular wall (Sample-2) and 100 m away to circular wall (Sample-3)(Fig.1). The number of colony forming units obtained per one ml were 5800, 8000 and 5800 in sample -1, 2 and 3 respectively. The culturable bacterial load obtained per ml of sample -2

was significantly higher to that of sample-1 and 3. The strains collected from all the three samples were analysed to get average resistance to each of the drug used in this study. On an average 6,500 colony forming units were obtained per ml of water samples at this location. Out of which 39.8% were found resistant to Ampicillin, 0% to Kanamycin, 11.9% to Tetracycline, 7.9% to Chloramphenicol, 3.5% to Cefotaxime, 7.4% to Cefepime, 10.9% to Ceftazidime, 55.5% to Eartapenem, 47.1% to Cefpodoxime and 0% to Ciprofloxacin. The resistant pattern is shown in Fig.13A.

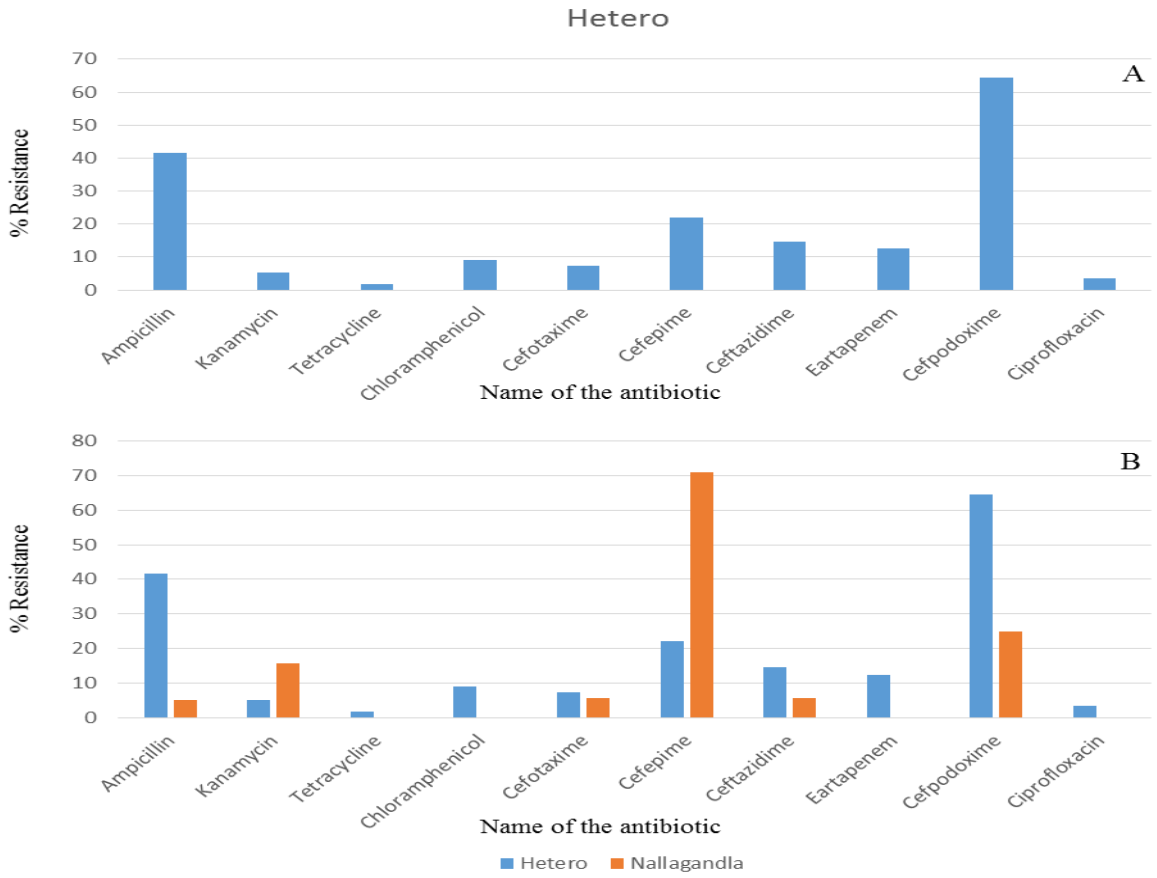

Fig. 13: Comparison and evaluation of drug resistance pattern between water samples collected Near Hetero-Kazipally (A) and water from Nallagandla lake taken as control (B).

The resistance towards Ampicillin, Tetracycline, Chloramphenicol, Ceftazidime, Eartapenem and Cefpodoxime in water samples collected from

this location are significantly higher than the controls whereas Kanamycin, Cefotaxime and Cefepime resistance is significantly lower than the bacterial resistance obtained at Nallagandla lake. Interestingly in cfu's found at both control and Hetero no resistance was seen against ciprofloxacin (Fig.13B).

Gaddapotharam (Seepage):

The culturable bacterial load obtained per ml of water samples collected from in samples -1 located inside circular tank, spot-2 which represents outer channel of circular tank and spot-3 representing drain outside the circular tank (Fig.1) were 6,80,000, 84,000 and 3,800 cfu's respectively. The cfu's obtained at spot-1 is highest as the water is stationary and has scope to retain all the bacteria that propagate using organic matter found in the tank. Rest of the spots, 2 and 3 indicate bacterial load found in in-flow and the outlet.

The data collected from these 3 spots was used to generate an average cfu's resistance to various antibiotics and represented in the form of a graph (Fig.8A). At this location cfu's showing resistance to Ampicillin was 41.6%, 5.2% to Kanamycin, 1.8% to Tetracycline, 9.2% to Chloramphenicol, 7.3% to Cefotaxime, 22% to Cefepime, 14.5% to Ceftazidime, 12.5% to Eartapenem, 64.4% to Cefpodoxime and 3.5% to Ciprofloxacin. The highest resistance is seen towards eartapenem and lowest to Tetracycline (Fig.14A). The resistance towards Ampicillin, Tetracycline, Chloramphenicol, Cefotaxime, Ceftazidime, Eartapenem, Eefpodoxime and Ciprofloxacin in water samples collected from this location are significantly higher than the controls. However cfu's obtained from Nallagandla lake showed more resistance to Kanamycin and Cefepime than the counter parts obtained at water from seepage (Fig.14B).

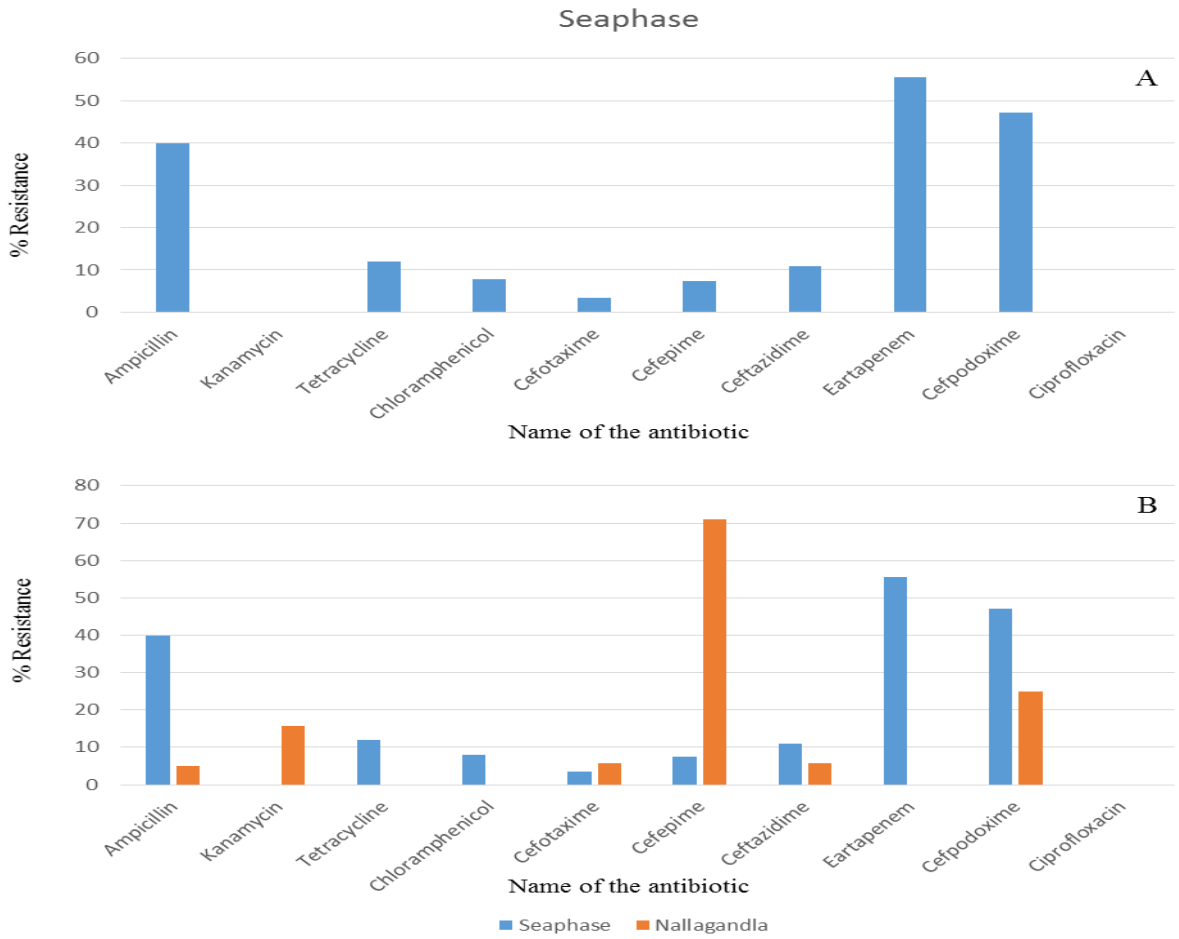

Fig. 14: Comparison of drug resistance pattern between water samples collected at Seepage-Gaddapotharam (A) and Nallagandla lake taken as control (B).

Jadcherla-SEZ (Aurobindo)

The culturable bacterial load obtained per ml were calculated at each spot using the colony forming units obtained from an appropriate dilution factor. The cfu obtained from spot- 1 which represents the drain outlet, spot- 2 representing the side line near road, spot- 3 representing inside the unit and spot- 4 which represents the rain harvesting pit (Fig.1) were found to be 15,000, 18,000, 25,800 and 19,000 cfu's respectively.

A graph was plotted taking average of resistant strains obtained from all the spots at this location. It is observed that, of an average bacterial load of 19,450 cfu's obtained per ml in this location, 38.5% were resistant to Ampicillin,

11.8% to Kanamycin, 11.3% to Tetracycline, 28.9% to Chloramphenicol, 15.8% to Cefotaxime, 32.3% to Cefepime, 17.2% to Ceftazidime, 23.3 % to Eartapenem, 73.2% to Cefpodoxime and 13.3% to Ciprofloxacin. The highest resistance is seen towards Cefpodoxime and lowest to Tetracycline (Fig.15A). The resistance obtained at this location was then compared with that of the data generated from control water samples collected from Nallagandla lake (Fig. 15B). As shown in Fig. 15B more resistant colonies were found for Cefepime in bacteria isolated from Nallagandla lake. However for Cefpodoxime and Ampicillin more resistant colonies were found at Aurobindo unit VII (15B).

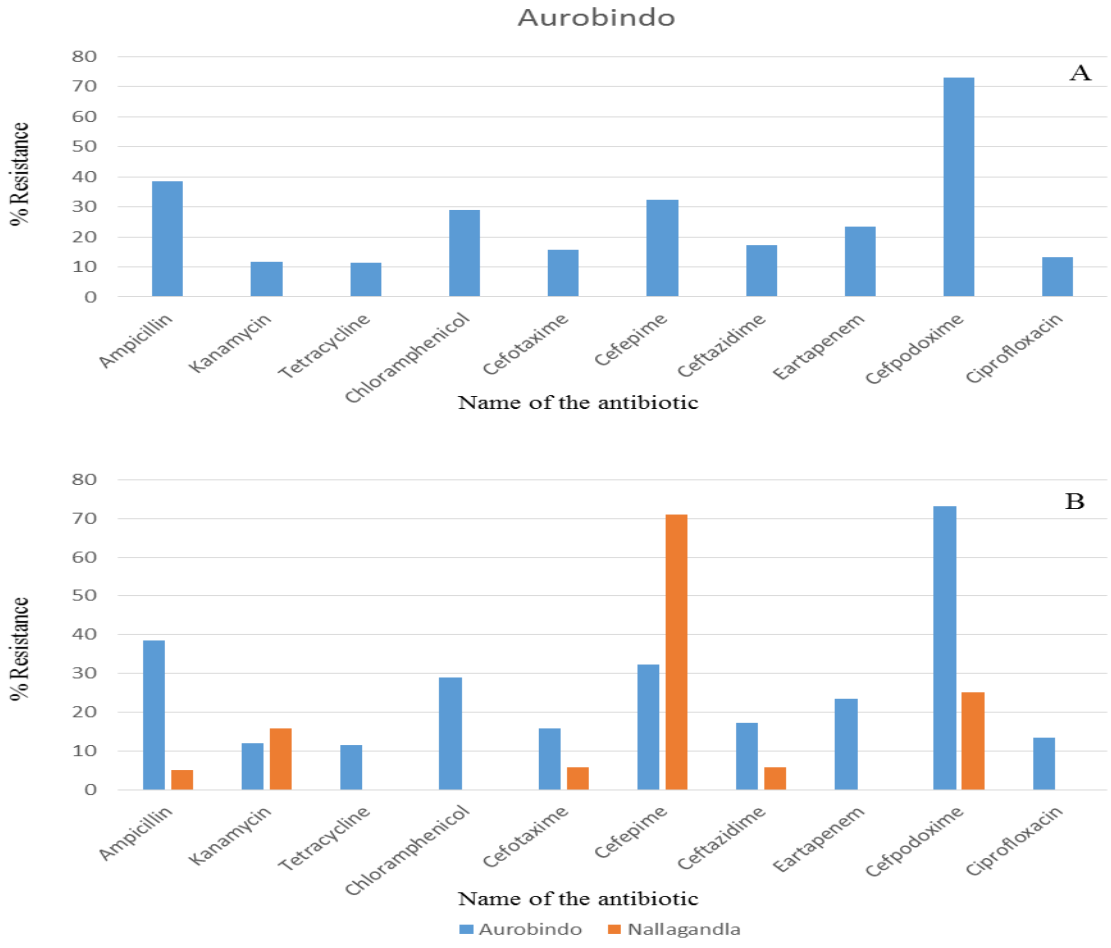

Fig. 15: Comparison of drug resistance pattern between water samples collected from Aurobindo unit VII (A) and water samples from Nallagandla Lake taken as control (B).

Observations:

The drug resistance patterns for samples collected from Isnapur lake was compared with resistance pattern from Nallagandla lake, which is located about 50 km away from Isnapur Lake. The resistance pattern is same between the two lakes. However the resistance to Ampicillin and Cefpodoxime are more in Isnapur lake whereas kanamycin and Cefepime are lower compared to bacterial resistance obtained at Nallagandla lake (Fig. 10B). Since there is no indication of drug manufacturing units in the vicinity of Nallagandla lake, it is taken as control sample. Comparison of resistance pattern reveals interesting results. There exists no evidence to show that PMCs has selectively enriched bacteria to a particular antibiotic.

Inference:

- Culturable bacteria represented both gram negative and positive bacteria.
- Drug resistant Bacterial Strains were found both in samples collected from PMCs and outside of PMCs
- The samples collected from Nallagandla lake have more or equal number of drug resistant strains for certain antibiotics than in samples collected around PMCs.
- Resistant bacterial strains were found, more or less in equal number, in samples collected 1 and 5 KM upstream of the Jadcherla SEZ, which is a non-industrial area.
- No clear evidence of selective enrichment is observed among samples collected from PMCs.

Further Studies:

The isolated resistant strains are being used to determine their taxonomic identity. Chromosomal DNA from all the resistant bacterial strains were isolated to amplify 16SrRNA gene. The amplicon containing 16SrRNA gene is being sequenced. After obtaining the sequence the same will be used to generate Dendrogram to establish the taxonomic status of the resistant bacteria. This studies are being extended for the resistant strains isolated both from control samples and resistant strains isolated from the PMCs. This study will through light on nature of resistant strains and selective enrichment of pathogenic bacteria near PMCs.