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ANTIBIOGRAM OF *ACINETOBACTER SPECIES* ISOLATED FROM VARIOUS CLINICAL SAMPLES

Jadhav Manisha Gorakh*[1], Pawar Sunil Trimbakrao[2], Karad Dilip Dashrath[3]

[1*]Department of Microbiology and Research Center, Shri Shivaji Mahavidyalaya, Barshi, Solapur, Maharashtra, India.

[2]Tuljaram Chaturch and College, Baramati, Dist-Pune, Maharashtra, India.

[3]Post Graduate Department of Microbiology and Research Center, Shri Shivaji Mahavidyalaya, Barshi, Solapur, Maharashtra, India.

ABSTRACT

Acinetobacter baumannii is a vital opportunistic pathogen to high virulence which is responsible for severe nosocomial infections. These species of the bacterium, particularly multi-drug resistant strains has been involved as the cause of serious infectious disease in various parts of the hospitals. Therefore, the treatment of such infections due to their broad resistance to different antibiotics is difficult. Additionally, since environmental factors and the several patterns of antimicrobial agents play an important role in the creation and expansion of these strains in different parts of the world, in the present study, the antibiogram of multi-drug resistant species of Acinetobacter from clinical samples was performed. The strains were identified on the basis of cultural, morphological characterizations, by 16s rRNA gene sequencing and VITEK-2 analysis. After identifying the organism up to species level, in order to determine the sensitivity of these isolates of Acinetobacter to 13 antibiotics, standard methods according to CLSI guidelines were performed. In the present study, resistance to three or more of three classes of antibiotics was considered as multidrug resistance was defined. In this study, total 60 Acinetobacter species and strains were isolated from patients. Of which, the antibiogram of two strains of Acinetobacter Viz. *Acinetobacter baumannii* MJ-142 and Acinetobacter baumannii MJ-143 was shown. Isolates of Acinetobacter showed the highest resistance to almost all the antibiotics used in the present study Viz. Meropenem (10µg), Cefepime (30µg), Amoxycillin (10µg), Gentamicin (10µg), Nitrofurantoin (300µg), Nalidixic acid (30µg), Tigecycline (15µg), Ampicillin (10µg), Imipenem (10µg), Amikacin (30µg), Ertapenem (10µg), Colistin (10µg) and Piperacillin (100µg). Multi-drug resistant Acinetobacters are growing and considered as a chief threat for hospitalized patients, as a result change in the consumption patterns of antibiotics and the control of hospital infections appears to be necessary.

KEYWORDS

Acinetobacter, Antibiogram, Multi-drug resistance and Nosocomial infection.

Author for Correspondence:
Jadhav Manisha Gorakh,
Department of Microbiology and Research Center,
Shri Shivaji Mahavidyalaya, Barshi,
Solapur, Maharashtra, India.

Email: manisha4feb@gmail.com

INTRODUCTON

Acinetobacter spp. arises naturally in many various habitats which include soil, food and surface waters. Acinetobacter poses an increasing health problem, causing different infections having limited to no antibiotic therapeutic options left. The members of the genus Acinetobacter are omnipresent, free-living and saprophytic bacilli which can be obtained

easily from soil, food, water and sewage (Dougari *et al*, 2011)[1]. These are non-fermented of glucose, gram-negative, aerobic and opportunistic pathogens that emerge as a significant cause of hospital-acquired infections and intermittent outbreaks globally. It's most common and important representative is Acinetobacter baumannii and the other species such as Acinetobacter johnsonii, *Acinetobacter lwoffii* and *Acinetobacter* haemolyticus are rarely isolated from patients (Peleg A P *et al*, 2008)[2]. Its great capacity to survive in the low-moist environment with its ability to develop resistance to different antimicrobial agents can increase the possibility of spreading in hospitals *(*Yu Yu *et al*, 2004)[3]. The most striking characteristic of Acinetobacter spp. is their natural resistance to several antibiotics and the ability to simply develop new resistances under antibiotic pressure. Consequently, *Acinetobacter baumannii* in particular has become one of the problematic nosocomial pathogens. In this study, Acinetobacter spp. was isolated from clinical samples. Susceptibility testing was carried out for 13 clinically relevant antibiotics.

Now-a-days, due to *Acinetobacter baumannii* significant clinical properties coupled with its ability to achieve drug resistance is considered as one of the microorganisms that threaten antimicrobial medication. *Acinetobacter baumannii* causes nosocomial infections such as urinary tract infections (UTI), bacteremia and secondary meningitis, but it has prominent role in the creation of hospital pneumonia especially pneumonia which acquired in upper respiratory tract in patients that hospitalized in intensive care units (ICU) (Villers *et al*, 1998[4], Anstey *et al*, 2002[5], Fournier and Richet, 2006[6], Dijkshoorn *et al*, 2005[7], Rizos *et al*, 2007[8], Peleg *et al*, 2008[2]).

Different studies demonstrated that a range of species of Acinetobacter baumannii are resistant to broad range of antibiotics. Spread of multi-drug resistant Acinetobacter baumannii is not limited to hospitals of one city but is also vitalin national scale (Unal *et al*, 2005[9], Metan *et al*, 2007[10], Wroblewska *et al*, 2007[11], Wisplinghoff *et al*, 2007[12]). Since various factors causing resistance in *Acinetobacter baumannii*, treatment of infections caused by this organism should be based on the perfect antibiotics sensitivity tests, that's why having information concerning the prevalence and pattern of bacterial resistance to such drugs is important (Van Dessel *et al*, 2004[13], Scott *et al*, 2007[14], Halstead *et al*, 2007[15]). In the present study, investigation of multi-drug resistance in Acinetobacter baumannii strains was isolated from clinical samples was done.

MATERIAL AND METHODS

Isolation of *Acinetobacter baumannii*

These enriched peptone cultures were transferred on MacConkey's agar plate and Blood agar plate which was used as the selective media. By using the four quadrants square culture method these peptone water cultures were then streaked onto sterile agar plates. All these plates were incubated at 37ºC in an incubator. After 24 hours results were recorded. After incubation, colonies showing different morphological mucoid characteristics were then selected for further study.

Identification of Acinetobacter baumannii

The present bacterial isolate was identified by microscopic, culture, microscopic and biochemical characterizations tests, 16s rRNA analysis and also by using the Vitek-2 system auto analyzer. Using biochemical tests, PIB Win software also used for identification. (Bryant, 2004)[16].

Antibiotic Sensitivity Testing

Sterile Mueller Hinton agar plates were prepared. Sterile nontoxic cotton swab which was previously dipped into the inoculums was soaked firmly by rotating against the upper inside wall of the tube for to remove the excess fluid. The complete agar surface of the plate was spreaded with sterile swab three times by turning the plate at 60º angles. The octadisc were put on using aseptic inoculation technique. These plates were kept in a refrigerator at 2ºC to 8ºC for 30 minutes. After that, the plates were further incubated at 35ºC and examined after 16 to 18 hours. The zones showing complete growth inhibition were measured and recorded the diameters of zones to the nearest millimeter, using standardized instruments or zone scales.

Antibiotic Susceptibility testing was carried out for 13 types of antibiotics were performed by using the Disc Diffusion method on Mueller Hinton agar according to the instructions of the Clinical and Laboratory Standards Institute 2012. Discs of Meropenem (10µg), Cefepime (30µg), Amoxicillin (10µg), Gentamicin (10µg), Nitrofurantoin (300µg), Nalidixic acid (30µg), Tigecycline (15µg), Ampicillin (10µg), Imipenem (10µg), Amikacin (30µg), Ertapenem (10µg), Colistin (10µg) and Piperacillin (100µg) were used.

Any bacterial strain which resist to a minimum at least 3 different classes of antibiotics it is regarded as MDR. This method was performed according to CLSI.

Detection of plasmids by agarose gel electrophoresis

Plasmid isolation of transconjugants was done by Sambrook and Maniatis, 1989[17] method described above and detected by electrophoresis.

Curing of plasmid from bacteria

Curing of plasmids was performed by using method describe by Tomoeda *et al*, (1968)[18]. The 3 different conc. of ethidium bromide: 75µg/ml, 100µg/ml and 125µg/ml were used. Both the isolates MJ-142 and MJ-143 were selected for plasmid curing.

An overnight cultures of each of test organism in Luria Broth (LB) containing tetracycline for isolate MJ-142 and streptomycin for isolate MJ-143 respectively, each isolates were diluted to 10^4cells/ml using freshly prepared sterile LB by serial dilution technique. From this diluted culture, 0.5ml was added with 4.5ml LB containing different conc. of curing agents. Thus, the conc. became 10^3cells/ml. The cultures were then incubated at 37^{o}C in an orbital shaker at 150rpm for 48 hr. After incubation, the broth culture was again diluted to 10^3cells/ml with sterile normal saline and one ml of the culture was spread on Luria agar medium. After 24 hr. incubation at 37^{o}C, the plates were observed for growth. From plates, well-isolated colonies were randomly selected and simultaneously patched with a sterile toothpick on Luria agar medium without antibiotic and another Luria agar containing tetracycline and streptomycin, with a numbered grid line attached on the bottom of each plate. After 24 hr. incubation at 37^{o}C, plates were observed for the cured cells. The cured plasmid cells were detected by comparing the development of bacterial colonies on an antibiotic-containing plate with that of the normal (without antibiotic) plates. The samples that showed colonies on normal LB agar but failed to grow on LB agar supplemented with respective antibiotic were the possible cured isolates. Plasmid curing potential of each agent was then determined by calculating plasmid curing frequency (Trevors, 1985)[19].

RESULTS AND DISCUSSION

Colony Characterizations of Promising isolates

In the present study, colonies were in between 1 to 2mm in diameter. All isolates were circular to irregular in shape, while margins of all colonies were entire. The colonies exhibited convex and raised elevation. The colonies of the isolates were opaque and translucent. Colonies were pink to faint pink in colour. The specific types of colony characterizations shown by the bacteria will help for the identification as well as focus on the effective treatment against pathogens.

Biochemical Characterizations

The suspected colonies of Acinetobacter baumannii isolated were further identified through the biochemical tests (Barrow and Feltham, 2003)[20] using the standard procedures.

A series of morphological, physiological and biochemical tests were performed to identify the suspected *Acinetobacter baumannii* isolates. The test included Gram nature, motility, Oxidase activity, Catalase production, Oxidation-Fermentation (OF) test, Glucose fermentation, Lactose fermentation, Xylose fermentation, sucrose fermentation, Mannitol fermentation, Galactose fermentation, Mannose fermentation, Rhamnose fermentation, Voges-Proskauer (VP) test, Methyl Red (MR) reaction, Citrate Utilization, Urea hydrolysis test. All the tests were conducted according to the Bergey's Manual of Determinative Bacteriology (Bergey, 2005)[21].

In the present study, the biochemical characterizations were carried out in the Microbiology laboratory and the bacterial isolates

were identified up to Genus level and species level by referring Bergey's Manual of Determinative Bacteriology and 16s rRNA gene sequencing respectively. The results of biochemical characterizations of the present isolates are shown in the Table No.1.

Identification of the Promising Isolates

Identification of the promising isolates Viz. MJ142 and MJ143 was done by morphological, colony and biochemical characterizations, 16s rRNA analysis and VITEK-2 analysis.

Nucleotide Sequence Accession Numbers

The data of nucleotide sequence of MJ142 and MJ143 accounted in the present study have been submitted to the DNA Data bank of Japan (DDBJ) sequence database and assigned accession numbers LC491429 and LC491430 respectively.

Antimicrobial Susceptibility test

The susceptibility to the antimicrobial agents was determined by using the Disc Diffusion method (Bauer *et al*, 1959)[22]. The antimicrobial agents used were: Meropenem (10µg), Cefepime (30µg), Amoxycillin (10µg), Gentamicin (10µg), Nitrofurantoin (300µg), Nalidixic acid (30µg), Tigecycline (15µg), Ampicillin (10µg), Imipenem (10µg), Amikacin (30µg), Ertapenem (10µg), Colistin (10µg) and Piperacillin (100µg).

It is found from the Figure No.3 that the promising isolate MJ142 shows the largest inhibition zone diameter i.e. 15mm to nitrofurentoin and gentamicin followed by ampicillin, tigecycline, amikacin, amoxycillin, cefepime and colistin which is of 09mm, 08mm, 07mm, 07mm, 04mm and 03mm in diameter.

Furthermore, the largest zone of growth inhibition shown by the MJ143 is for Gentamicin which was followed by amikacin, cefepime, meropenem, imipenem and nitrofurentoin, colistin and tigecycline are 14mm, 10mm, 08mm, 07mm, 07mm, 06mm, 05mm and 04mm respectively. Whereas no zone of growth inhibition is shown by the MJ142 and MJ143 to meropenem, nalidixic acid, imipenem, ertapenem and piperacillin and amoxycillin, nalidixic acid, ampicillin, ertapenem and piperacillin respectively.

It is evident from the Figure No.3 that, in the present study, most of the *Acinetobacter spp.* were highly resistant to Meropenem, Nalidixic acid, Imipenem, Ertapenem and Piperacillin and Amoxycillin, Nalidixic acid, Ampicillin, Ertapenem and Piperacillin respectively by MJ142 and MJ143. The highly resistant patterns of the above the MJ142 and MJ143 to the drugs used in the present study indicate that they are not effective drugs for the same. Both the promising isolates were very resistant to the antimicrobials used in the present study.

Plasmid Isolation, Curing and Gel Electrophoresis

Plasmids are the extra chromosomal elements which are responsible for the development and spread of antibiotic resistance in *Acinetobacter species.* Isolation of plasmid DNA from Acinetobacter baumannii MJ-142 and *Acinetobacter baumannii* MJ-143 isolates revealed the presences of single plasmid. The size of the isolated plasmid was approximately more than 22 kb (Figure No.4).

Discussions

Acinetobacter strains with antibiotic resistance have been reported from all around the world (Brink *et al*, 2007)[23]. Hujer *et al*, (2006)[24] showed in their study which was conducted on civilian and military patients in Iraq and Afghanistan reported that 15% of the strains were resistant to all the nine antibiotics which were tested and 89% of strains displayed resistance to at least three antibiotic classes. In their study they showed that more than 90% of *Acinetobacter* isolates were resistant to ciprofloxacin, whereas less than 80% to cephalosporins with a broad spectrum, 81% to at least one of the amino glycosides (tobramycin or amikacin), 20% to imipenem and 40% to ampicillin-sulbactam. In a study performed by Ayan and colleagues in year 2003 that, they have studied the 52 strains, all were resistant to cefepime, piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, ticarcillin-clavulanic acid, gentamycin, ceftriaxone and resistant to ciprofloxacin, tobramycin, co-trimoxazole, ampicillin-sulbactam and amikacin. These results highly conform to the results of our study.

Rahbar and colleagues (2010)[25] were determined that, *Acinetobacter baumannii* shows high percentage of resistance to ceftriaxone (90.9%), piperacillin (90.9%), ceftazidime (84.1%), amikacin (85.2%) and ciprofloxacin (90.9%) that results partly conform with results of this research. They had also conducted that imipenem was the most effective agent against these organisms (resistance 4.5%) that is in conflict with our results (Rahbar *et al*, 2010)[25].

Karlowsky *et al*, (2003)[26] demonstrated in their study that 90% of *Acinetobacter baumannii* strains were sensitive to meropenem, but in this investigation, both the strains showed resistance to meropenem and usage of this antibiotic with tobramycin which could be more effective against *Acinetobacter baumannii* strains.

In a study that conducted by Hoe Koo *et al*, in 2007 to 2008[27], they showed amikacin as the most effective drug among nine antimicrobial agents used, contrasting, in the present study Nitrofurentoin and Gentamycin were the most effective agents among 13 antimicrobial agents used in the study.

Overall results indicate that among the common Acinetobacter species Viz. *Acinetobacter baumannii MJ-142* and *Acinetobacter baumannii MJ-143* is more responsible for nosocomial infections which are multi-drug resistant, so control of hospital infections seems to be necessary which were investigated. With this regard, in response to the uncontrolled use of antibiotics, multi-drug resistant *Acinetobacter baumannii* n hospital environment increased, so control of antibiotics usage in hospitals play an significant role in preventing the emergence of such strains and infections caused by them.

Table No.1: Biochemical characterizations of the present isolates MJ142 and MJ143

S.No	Characters	MJ142	MJ143
1	Gram nature	Non-motile	Non-motile
2	Motility	Non-motile	Non-motile
3	Morphology	Coccobacilli	Coccobacilli
4	Oxidation-Fermentation (OF) test	O	O
5	Catalase	+	+
6	Oxidase	-	-
7	Glucose	+	+
8	Lactose	+	+
9	Xylose	+	+
10	Mannitol	-	-
11	Sucrose	-	-
12	Galactose	+	+
13	Mannose	+	+
14	Rhamnose	+	+
15	Citrate Utilization	+	+
16	Urea hydrolysis test	-	-
17	Methyl Reel	-	-
18	V-P Test	-	-

Table No.2: Antibacterial activity of different antibiotics and there diameter of zone of inhibition (mm)

S.No	Name of Antibiotics (concentration)	Different species of *Acinetobacter baumannii* Diameter of zone of Inhibition (mm)	
		MJ142	**MJ143**
1	Meropenem	00	07
	MRP-10	R	R
2	Cefepime	04	08
	CPM-30	R	R
3	Amoxycillin	07	00
	AMX-10	R	R
4	Gentamicin	10	14
	GEN-10	R	I
5	Nitrofurentoin	15	06
	NIT-300	I	R
6	Nalidixic acid	00	00
	NA-30	R	R
7	Tigecycline	08	04
	TAC-15	R	R
8	Ampicillin	09	00
	AMP-10	R	R
9	Imipenem	00	07
	IPM-10	R	R
10	Amikacin	07	10
	AK-30	R	R
11	Ertapenem	00	00
	ETP-10	R	R
12	Colistin	03	05
	CL-10	R	R
13	Piperacillin	00	00
	PI-100	R	R

Table No.3: Antibacterial activity of different antibiotics and there diameter of zone of inhibition (mm) after curing of MJ142 and MJ143

S.No	Name of Antibiotics (concentration)	Different species of *Acinetobacter baumannii* Diameter of zone of Inhibition (mm)	
		MJ142	**MJ143**
1	Meropenem	10	12
	MRP-10	S	S
2	Cefepime	08	10
	CPM-30	S	S
3	Amoxycillin	12	07
	AMX-10	S	S
4	Gentamicin	12	14
	GEN-10	S	S
5	Nitrofurentoin	18	12

	NIT-300	S	S
6	Nalidixic acid	10	12
	NA-30	S	S
7	Tigecycline	12	10
	TAC-15	S	S
8	Ampicillin	14	10
	AMP-10	S	S
9	Imipenem	10	12
	IPM-10	S	S
10	Amikacin	13	12
	AK-30	S	S
11	Ertapenem	10	08
	ETP-10	S	S
12	Colistin	08	10
	CL-10	S	S
13	Piperacillin	10	12
	PI-100	S	S

Phylogenetic Analysis of *Acinetobacter baumannii MJ-142*

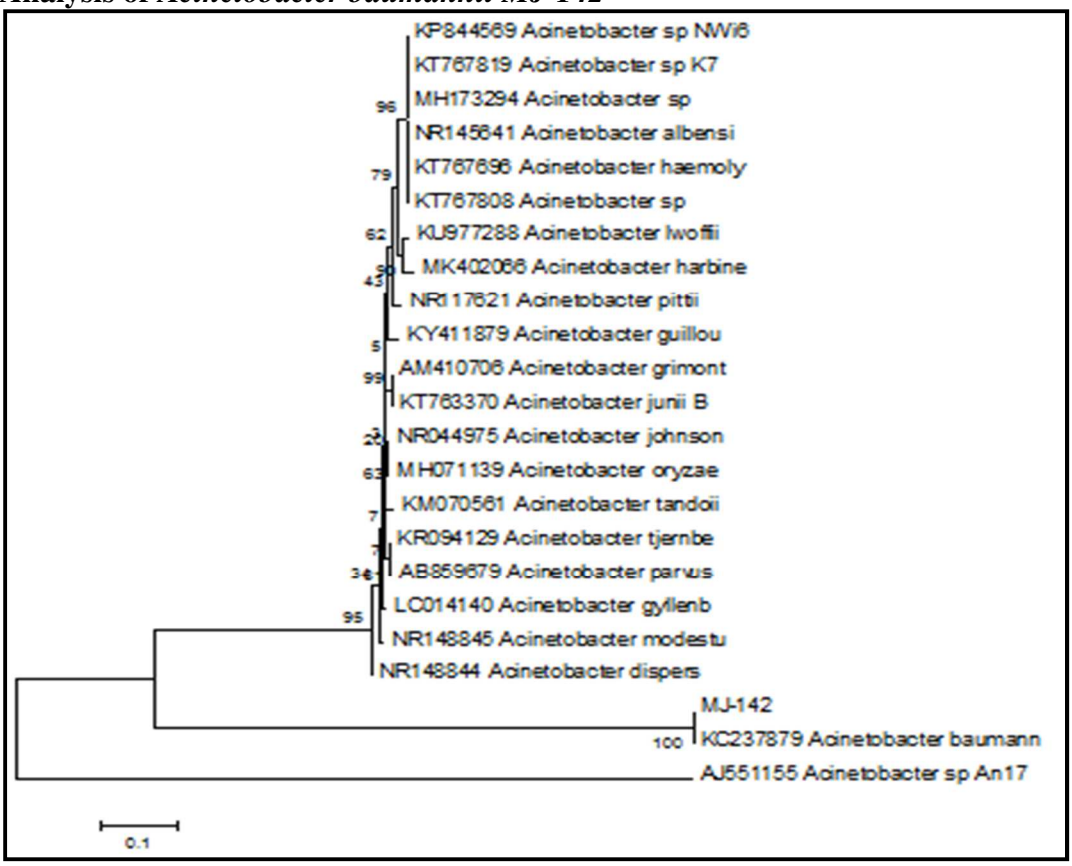

Figure No.1: Phylogenic tree of *Acinetobacter baumannii MJ-142*. *Phylogenetic* analysis of 16s rRNA gene sequence of *Acinetobacter baumannii MJ-142*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.1) indicates the genetic distance

Phylogenetic Analysis of *Acinetobacter baumannii MJ-143*

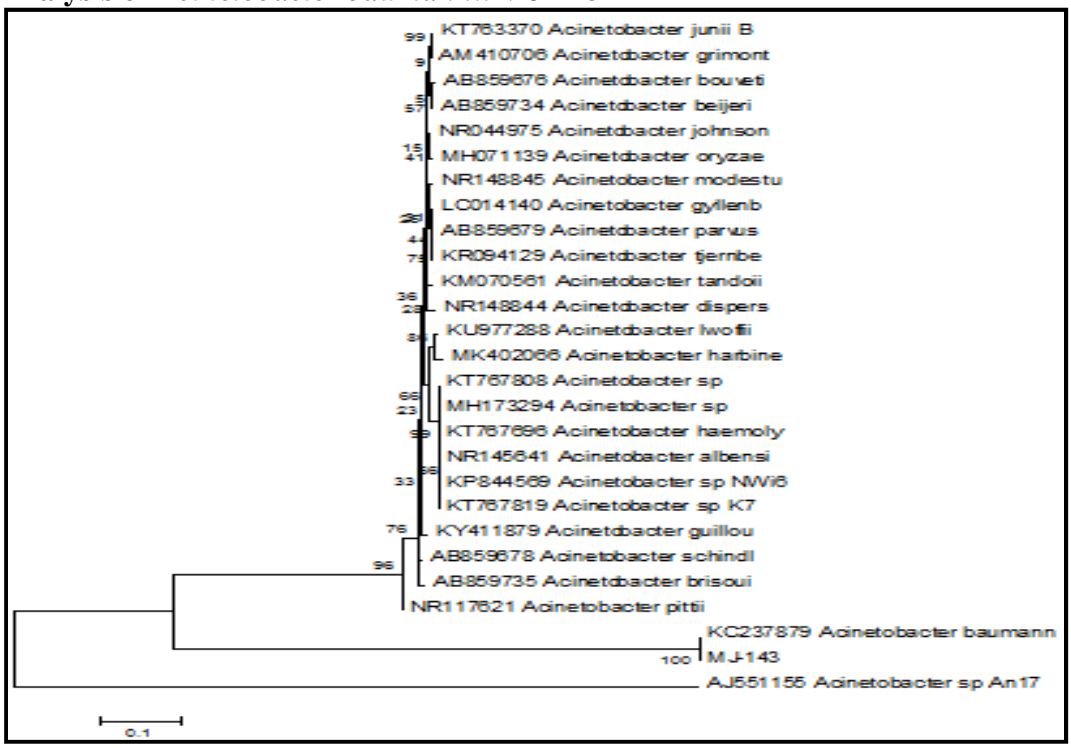

Figure No.2: Phylogenic tree of Acinetobacter baumannii MJ-143. Phylogenetic analysis of 16s rRNA gene sequence of *Acinetobacter baumannii MJ-143*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.1) indicates the genetic distance

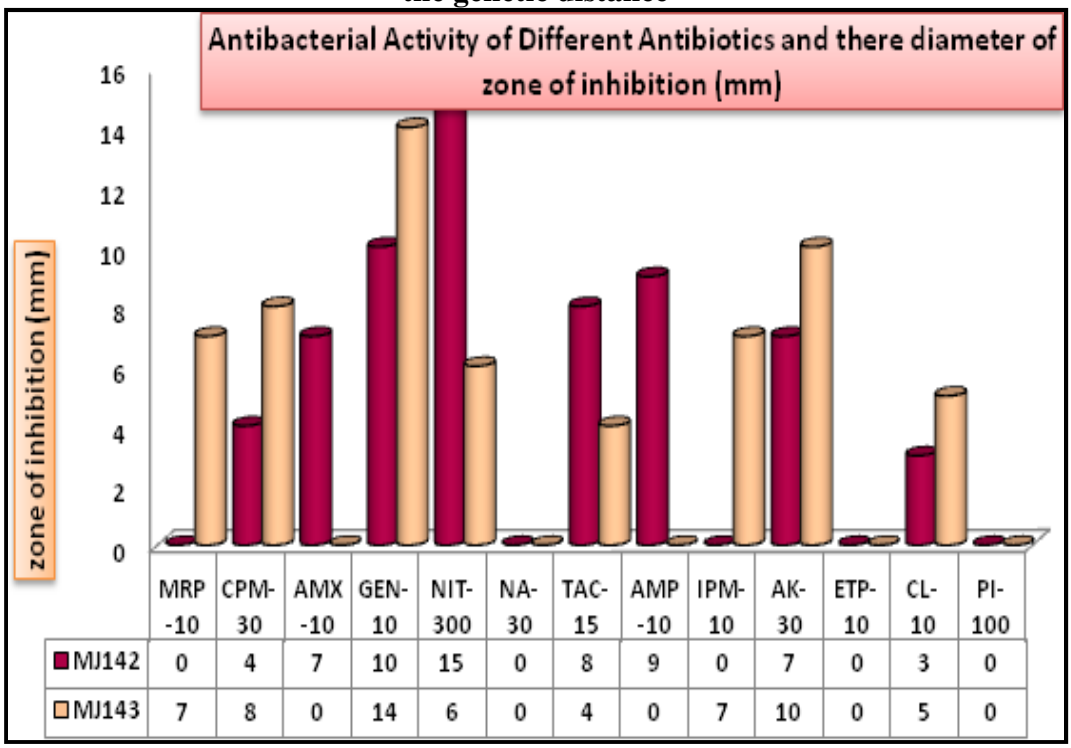

Figure No.3: Antibacterial Activity of Different Antibiotics and their diameter of zone of inhibition (mm)

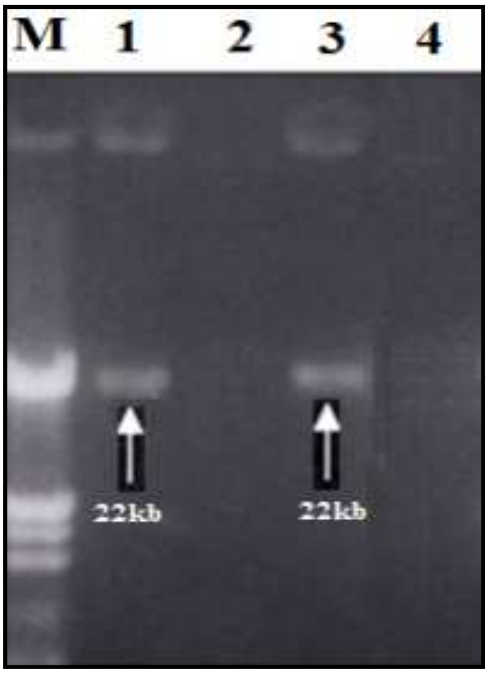

Figure No.4: Plasmid DNA profile of (Lane 1) *Acinetobacter baumannii MJ-142* (Lane 2) *Acinetobacter baumannii MJ-142* Cured (Lane 3) *Acinetobacter baumannii MJ-143* and (Lane 4) *Acinetobacter baumannii MJ-143* cured and (Lane M) DNA lambda EcoR1

CONCLUSION

We found both of *Acinetobacter* isolates were MDR. Elderly age, longer duration of stay, associated co-morbidity, being inpatients and invasive procedure were found to be the risk factors in the setup investigated. To avoid resistance, antibiotics should be exercised judiciously and empirical antibiotic therapy should be established for hospital according to the resistance rates of that center which should be regulated as per the results of antibiogram.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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