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Molecular and Antibiotic Resistance Study of Acinetobacterbaumannii Isolated From Different Sources

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Abstract

A total of thirty five *Acinetobacterbaumannii*i solates were identified from burn, wound, urine, and sputum samples among two hundred forty five patients admitted to Rizgary, Erbil Teaching, and West Emergency hospitals in Erbil city during the period of February 1, 2013to May 15, 2014. The isolateswere identified by colonial appearance, morphological characteristics, biochemical tests, VITEK 2 system, and Polymerase Chain Reaction (PCR) technique, through amplification of *bla*OXA-51. The PCR product on gel electrophoresis was 353bp which confirm that the isolates were *Acinetobacterbaumannii*. *A. baumannii*tested for antibiotics susceptibility test using agar diffusion method and the results showed that 35 (100%), 35 (100%), 25 (71.42%), 22 (62.85%), 19 (54.28%), 16 (45.71%), 14(40%),8 (22.85),7 (20),and 3 (8.57) were resistant to Vancomycin, Penicillin, Cefotaxime, Ceftriaxone, Erythromycin, Doxycycline, Streptomycin, Gentamycin, Imipenem, and Cefazolinrespectively.All isolates of *A. baumannii* were susceptible to ciprofloxacin. The isolates were screened for the presence of carbapenem resistance–associated outer membrane protein gene (*carO* gene) and the results shows that 18 (51.42%) isolates were positive for the (*carO* gene) using polymerase chain reaction (PCR) assay.To control the antibiotic resistance of the tested *Acinetobacterbaumannii*isolates, curing of plasmid DNA was conducted using ethidium bromide. One of the most resistance isolate was chosen for this purpose (A33) then treated with Ethidium bromide at concentration (50, 75,100,125,150,and 175µg/ml). The results revealed that the genes encoded resistance for Imipenem, Vancomycin, penicillin,and doxycycline were cured from A33 and the percentage of curing was (36.3%) and the best concentration was 125 µg/ml.

Introduction

Acinetobacterbaumannii is an opportunistic pathogenthat is frequently involved in outbreaks of infection, occurring mostly in intensive care units [1]. *A. baumannii* is mostly a cause of septicemia, pneumonia and urinary tract infection following hospitalization of patients with more severe illness. The constant increase in the antibiotic resistance of clinical bacterial strains has become an important clinical problem [2]. The accelerated emergence of antibiotic resistance among the prevalent pathogens is the most serious threat to the management of infectious diseases [3].Multidrug-resistant isolates of *A. baumannii* have been reported increasingly during the last decade, probably as a consequence of extensive use of antimicrobial agents in western countries [4]. Carbapenem resistance in this species is now observed increasingly worldwide, and constitutes a sentinel event for emerging antimicrobial resistance [5]. It is considered that resistance against carbapenem is, in itself, sufficient to define an isolate of *A. baumannii* as highly resistant [6]. *A.baumannii* contains a large circular chromosome, in addition to autonomous self-replicating cyclic DNA molecules, called plasmids. Among various plasmids present in bacterial strains R plasmids are very

significant as they confer resistance to one or several antibiotics and thus possess a threat to chemotherapy treatment of cells with certain chemical agents [7].Hence, controlling and elimination of the resistance that conferred by R-plasmid by using biological mutagen, physical and chemical or other curing agents will be quite useful to put out such resistance [8].Therefore,this study put light upon isolation and identification of *A. baumannii*from different clinical sources, performing antibiotic susceptibility test,detection of *carO*gene,and controlling antibiotic resistance using Ethidium bromide.

Materials and Methods

Specimen collection

From two hundred forty five patients admitted to Rizgary, Erbil Teaching, and West Emergency hospitals in Erbil city during the period of February 1,2013toMay 15, 2013,thirty five isolates of*A. baumannii* were identified using morphological,cultural,biochemicaltest,viteck 2system, in addition to PCR assay.

Molecular identification of A. baumannii

Extraction of genomic DNA fromA. baumannii isolates

Genomic DNA was extracted from all 35*A. baumannii*clinical isolates using a (InstaGeneTM Matrix - Biorad, US); this kit was designed for the isolation of DNA from whole blood, cultured cells, and bacteria. DNA was extracted by InstaGeneTM Matrix using bacterial protocol which summarized as follows: (InstaGeneTM Matrix Catalog 723-6030).

A well isolated bacterial colony from a blood agar plate was selected and suspended in 500µl of autoclaved water in a microfuge tube. The suspension was centrifuged for 1 minute at 10.000-12.000 rpm. The supernatant was removed. A 200 µl of InstaGene matrix was added to the pellet and incubated at 56°C for 15-30 minutes. InstaGene matrix should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. Vortexed at high speed for 10 seconds. The tube was placed in a 99°C heat block for 8 minutes. Vortexed at high speed for 10 seconds. Spinned at 10.000-12.000 rpm for 2-3 minutes. A 5µl of the supernatant was used per 50µl PCR reaction. The remiander of DNA was stored at -20°C until PCR analysis was carried out. If DNA didn`t use for a long period (1month) vortexed again for 10 seconds at high speed and spinned again for 2-3 minutes at 10.000-12.000 rpm.

PCR amplification

PCR was used to detect the OXA-51-like with amplicon size 353 bp in the genomes of the *A. baumannii*which is intrinsic for these bacteria. The OXA-51-likeprimers used were forward 5'-TAA TGC TTT GAT CGG CCT TG-3' and reverse:5'- TGG ATT GCA CTT CAT CTT GG-3',and PCR program was 94°C for 3 minutes, and then 35 cycles at 94°C for 45 seconds, at 57°C for 45 seconds, and at 72°C for 1 min, followed by a final extension at 72°C for 5 minutes [9]. PCR was performed in a 50µl of reaction volume. Master Mix 25 µl, forward Primer 5 µl, reverse Primer 5 µl, template DNA 5 µl, and sterile deionized water 10 µl.

Antibiotic Profile Test

Susceptibility of clinical isolates to 11 antibiotics (Mast, Merseyside, UK), including: Imipenem(IMP), Ceftriaxone(CRO), Ciprofloxacin(CIP) Gentamycin(GM), Cefazolin(CEFA), Vancomycin (V), Penicillin(P), Erythromycin(E), Streptomycin(S), Cefotaxime(CTX), and Doxycycline(DOXY) was evaluated by agar disk diffusion method on Mueller Hinton agar as recommended by Clinical and Laboratory Standards Institute (CLSI) [10].

Detection of carO gene in A. baumannii

PCR also was used for detection of *carO* gene in the genome of *A. baumannii* with amplicon size 186 bp in size. The primer used were forward 5'-CAGGTTACGGTGGTGCTTTG-3' and reverse 5'-GGTTAGTGCTTGCACCCCAT-3'.The PCR program was Initial denaturation (94°Cfor 3 min) was

followed by 30 cycles of amplification.Each cycle consisted of 94°C for 25 seconds, 52°Cfor 40 seconds, 72°C for 50seconds. A final extension step (72°Cfor 5 minutes) completed the amplification. PCR was performed in a 25µl of reaction volume. Master Mix 12.5 µl, forward primer 1 µl, reverse primer 1 µl, template DNA 1 µl, sterile deionized water 9.5 µl [11].

Agarose gel electrophoresis

To perform gel electrophoresis a method of [12] was followed with minor modifications. Agarose gel (1.5%) was prepared by adding 3g agarose to 200 ml 1X TBE buffer. The mixture melted in Microwave oven for 3-5 min or until becoming clear and well dissolved (not boiling). Left to cool to about 50°C. 4. Carefully 4µl ethidium bromide EtBr was added to agarose solution and mixed thoroughly by gentle swirling. The edges of tray sealed with the tape and the proper comb inserted into the tray. The agarose poured slowly into the tray, any bubbles pushed away to the side using disposable tip. The agarose allowed to solidify (15-30 min) at room temperature. The tape of the tray removed and the tray placed in the electrophoresis tank then the comb was removed. The electrophoresis tank filled with more TBE-buffer, so the gel is fully under buffer. PCR product with loading buffer loaded into the wells (15µl). The first well was used for the Ladder (3µl) (1 kb or 100 bp, depend on the size of the PCR product. The gel runs at 250V for 30 min. Finally, visualized under UV transilluminator and the gel photographed.

Plasmid Curing

Ethidium bromide as a curing agent

Curing by ethidium bromide was done by the procedure mentioned by [13]. Five ml of LB broth containing appropriate antibiotic at final concentration inoculated with single colony of*A. baumannii* isolate, incubated at 37^{o}C for 24 hours, after overnight 0.1 ml of the broth bacterial culture were inoculated into 5 ml LB broth containing (50,75,100,125,150,and 175 µg/ml) separately of ethidium bromide and incubated with shaking at 37^{o}C for 48 hours. Serial dilutions were prepared up to 10^{-6} for each concentration, 0.1 ml from the last three dilutions were spreaded onto nutrient agar plates and incubated at 37^{o}C for 24 hours. After incubation time, fifty colonies were transferred to nutrient agar plate and incubated over night at 37^{o}Crepresenting the master plate. Five colonies were randomly chosen, picked up and transferred to Muller Hinton agar plates and tested forantibiotic sensitivity pattern, incubated at 37^{o}C for 24 hours. After overnight incubation the percentages of curing colonies for both antibiotic sensitivity patterns were calculated.

Isolation of Plasmid DNA Content from A. baumannii

DNA spin™ Plasmid DNA Purification Kits (Intron/ Korea) procedure is based on alkaline lysis method of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt.

A single colony picked up from a freshly streaked bacterial plate and inoculated into LB plus appropriate antibiotic. The culture was incubated overnight with shaking.Three to five (3 – 5) ml of bacterial culture harvested by centrifugation at 13,000 rpm for 30 seconds. at room temperature then the supernatant were discarded.The pellet were resuspended in 250 µl of Resuspension Buffer, vortexed or pipetted until no clumps of the cell pellet remain.Two hundred fifty µl of Lysis Buffer were added to resuspended cells. The tube was closed and gently mixed by inverting the tube several times (not exceeded 5min for lysis time).Three hundred fifty µl of Neutralization Buffer were added and gently mixed by inverting the tube several times.Centrifugated at 13,000 rpm for 10 minutes at 4ºC during the centrifugation period, the column were inserted into the collection tube.After centrifugation, the supernatant transferred promptly into the column.Centrifugated at 13,000 rpm for 60 seconds the column removed from the collection tube, the filtrate discarded in collection tube. Then the spin column placed back in the same collection tube.Five hundred µl of Washing Buffer A were added and centrifugated at 13,000 rpm for 60 seconds. the column removed from the collection tube, the filtrate discarded, and the spin column placed back in the same collection tube. Seven hundred µl of Washing Buffer B were added, centrifugated at 13,000 rpm for 60 seconds. the filtrate discarded and the spine column placed back in the same collection tube. Centrifugated at 13,000 rpm for 60 seconds to dry the filter membrane. The columns were put into a clean and sterile centrifuge tube. Fifty µl of

Elution Buffer were added to the upper reservoir of the column, and left it standed for 1 minute. then, the tube centrifugated assembly at 13,000 rpm for 60 seconds.

Agarose electrophoresis technique [14]

Preparation of 1% agarose gel: The gel (1%) was prepared by dissolving 1 g of agarose powder in 100 ml of 0.5 X TBE (Tris base ethidium bromide) buffer, boiled until all agarose was dissolved and left to cool at 50 ºC, 8 µl of ethidium bromide was added, the gel was poured in to the glass plate that contained appropriate comb, the gel was left to solidify and the comb was removed gently, the gel was soaked in a gel tank containing TBE buffer should cover the surface of the gel.Tenµl of plasmid DNA samples were mixed with 5 µl of loading buffer, and the mixture was slowly loaded in to the wells on the gel; also a molecular weight marker was loaded as control.The electrophoresis apparatus was joined to power supply, turned on and the samples electrophoresed at 10 volt/cm for 1 hour.The gel was visualized by UV-transilluminator, and then photographed.

Results and discussion

Identification of A. baumannii isolates

It grows on CHROMagar*Acinetobacter*media in a temperature at 37^{o}C for 18-24 hours. The colonies appear strictly aerobic, 1.5 to 2.5 mm in diameter, and red color. Under the light microscope, *A. baumannii*appears as gram- negative, rod shape during rapid growth and coccobacillary shape in the stationary phase and occur in pairs. The biochemical tests for *A. baumannii*were positive for catalase, citrate, and indole, but they were negative for oxidase test, voges-proskauer, methyl red, glucose and lactose fermentation were positive, but H_2S production was negative on TSI. Variable reaction to urease production test, and they were non-motile. According to the results obtaining from these approaches, and depending on [15] and [16], 35 *A. baumannii*were isolated.

Identification of A. baumannii by VITEK 2 system

These formats are more focused on the clinical microbiology laboratory and provide increased levels of automation and capacity for higher volume laboratories, and consist of 48 biochemical tests. VITEK 2 system was performed to all isolated *A. baumannii*using classical approach, to support the results and to confirm that the isolates were *A. baumannii.* The profile bionumber of *A. baumannii*isolate A35, according to laboratory report1, the result of VITEK 2 system were identified *A. baumannii*complex (*A. baumannii- A. calcoaceticus*), but this system selected 99% as *A. baumannii*. To be sure that the identified isolates were *A. baumannii*the molecular technique (PCR System) was performed.

Molecular Identification of Acinetobacterbaumannii

To confirm that the identified bacterial species are *A. baumannii*species the molecular based method was performed through detection of *blaOXA-51* gene in the extracted DNA for all isolates, using the PCR technique by detection of the *OXA-51* carbapenemase gene. *OXA-51* gene are belong to the same group of naturally occurring oxacillinases in *A. baumannii,* this fact has been used to develop a PCR experiment with primers specific for this gene, which can be used as a simple and reliable way of identifying *A. baumannii* [17]. The PCR products for all 35 isolates of *A.baumannii* were positive for *blaOXA-51* gene and these results are shown in *Figure:1* that the amplified gene (353bp). The present results support those of a previous report, suggesting that *bla*OXA-51-like is ubiquitous in *A. baumannii.*[9]and[11] were used *blaOXA*-51for identifying*A. baumannii*. These results provide evidence that detection of *bla*OXA-51 can be used as a simple and reliable way for identifying *A. baumannii*. We have found *blaOXA-51* in all isolates of *A. baumannii*.

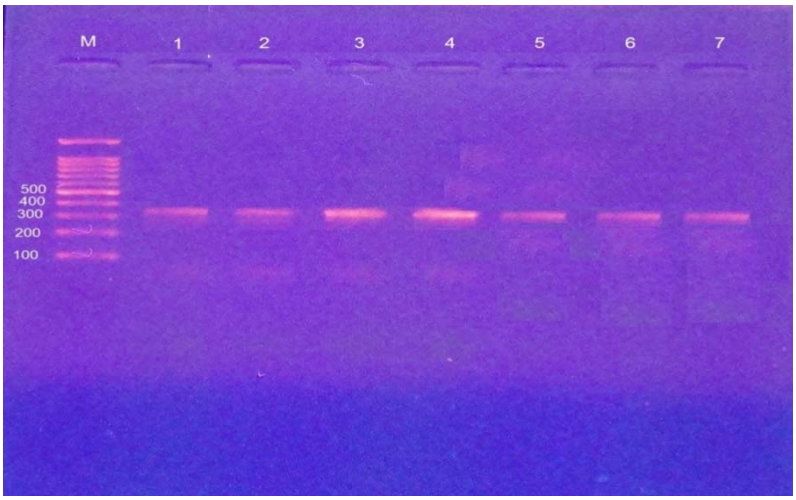

Figure- 1: Agarose gel electrophoresis of the PCR amplified *blaOXA-51* product. Lane M: ladder. Lane 1 to 7: Identified as *A. baumannii*and had *bla*OXA-51.

Antibiotic resistance profile

The antimicrobial susceptibility testing by agar disc diffusion method among *A. baumannii* isolates determined that the percentage of resistance to Vancomycin, Penicillin, Cefotaxime, Ceftriaxone, Erythromycin, Doxycycline , Streptomycin, Gentamycin, Imipenem , Cefazolin,andciprofloxacin were 35 (100%) , 35 (100%), 25 (71.42%), 22 (62.85%),19 (54.28%), 16 (45.71%), , 14(40%),8 (22.85%),7 (20%),and 3 (8.57%), and 0(0%) respectively (*Table: 1*).

Table- 1: Antibiotic Resistance Patterns of *A. baumannii*

Antimicrobes	Symbol	No. of resistance isolates	Resistance%
Vancomycin	V	35	100
Penicillin	P	35	100
Cefotaxime	STX	25	71.42
Ceftriaxone	CRO	22	62.85
Erythromycin	E	19	54.28
Doxycyline	DOXY	16	45.71
Streptomycin	S	14	40
Gentamicin	GM	8	22.8
Imipenem	IPM	7	20
Cefazoline	CEFA	3	8.57
Ciprofloxacin	CN	0	0

The highest rate of resistance among *A. baumannii* was related to Vancomycin and penicillin with 100% frequency while the lowest resistance was for Cefazolin with 8.57% frequency while all were sensitive to Ciprofloxacin.Similar results obtained by [18] who reported that the percentage of resistance to Vancomycin and penicillin with 100% frequency.Also [19] mentioned that all isolates appeared of *A. baumannii*were resistance to Vancomycin and penicillin. All isolates were showed great resistance against second generation of Cephalosporin (CTX and CRO). The results were 71% and 62% respectively. [18] found 68% and 56% of the isolates were resistance to these antibiotics. [20] referred that 69.9% and 58% of the isolates were resistance to the former antibiotics .These finding is consistent with our results. The resistance of *A. baumannii*to E was 54%, that near with [18] who reported 40%,while disagree with [19] who discovered that the resistance to sameantibiotic was 100%.On the other hand results showed that CIP is the most effective antibiotics against *A. baumannii* .Similar results obtained by [18], but disagree with [21] who found that 52 (86.6%) of the isolates were resistance to ciprofloxacin.[22] in their study that was conducted on military and civilian patients in Iraq and Afghanistan reported that 15% of the strains were resistant to nine antibiotics

which were tested and 89% of strains showed resistance to at least three antibiotic classes.Resistance patterns among nosocomial bacterial pathogens in hospitals may vary widely from country to country at any given point and within the same country over time [23]. Multi-drug resistance is rarely found in community isolates of *A. baumannii*. Whereas, the prevalence of the MDR *A. baumannii*among hospital isolates has increased [24]. Different mechanisms contributing to resistance including; carbapenemase production, modification of penicillin-binding proteins (PBPs), loss of porins, and/or altered efflux pump activity [25] and [26].Recent studies have shown that *A. baumannii*has emerged MDR and Pan drug resistant, i.e. isolates resistant to all antimicrobial agents and their prevalence can be incredibly high in Iraq, Middle East and Asia [27] and [28].Moreover,the high resistance of the bacterial isolates in this study to different antibiotics may be related to the presence and absence of plasmids within heterogeneous population of these bacteria and the number of plasmids in bacteria has more effect in the bacterial resistance.Inaddition to that,using inaccurate concentration of antibiotics or drug or unnecessary medicine leads to the resistance of sensitive bacteria, and resistance bacteria change to multi-resistance, in addition to weakling immune system in some human due to poor nutrition or heredity factors make bacteria to be more resisting [29].

Detection of carO gene among A. baumanniiisolates

Results in *Figure: 2* showed that *A. baumannii*isolates that contain carO primer, revealed positive PCR product on gel electrophoresis for 18 (51.24%) samples among 35 samples tested for the presence of *carO*gene.[30] were screened for carbapenem resistance-associated outer membrane protein (*CarO*) amongst Imipenemresistant *A.baumannii* isolates from ten hospitals in Western China using polymerase chain reaction (PCR).

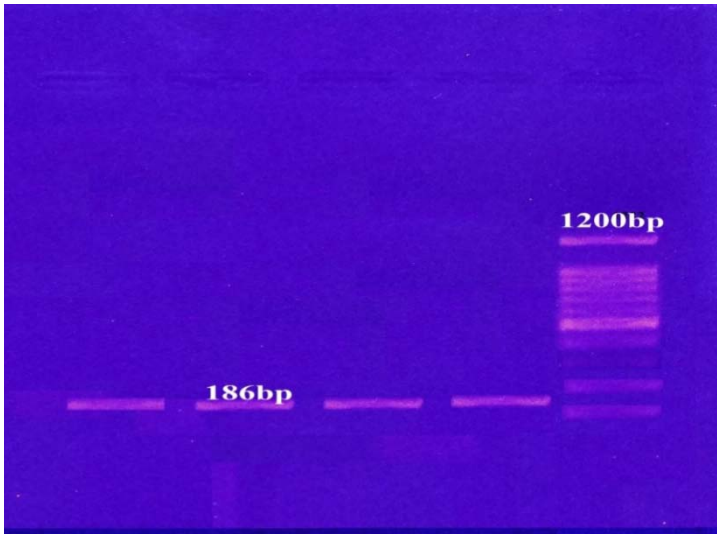

Figure- 2: Agarose gel electrophoresis of the PCR amplified *carO*geneproduct.

Amongst 272 Imipenem-resistant isolates, 5(1.8%) isolates harbored the *carO*gene.Our results lower than the finding of [31] who found that *carO* gene detected in 87 % of total *A.baumannii* isolates from china hospitals.On the other hand, our results disagreed with [21] who found in their study that *carO* gene was detected in 80% out of 60 *A. baumannii* isolates from different hospitals in Erbil city.Carbapenem remain the antibiotic of choice to treat *A. baumannii*and other Gram negative infections due to both a wider spectrum of antibacterial activity and less frequent side effects. However, their overuse and misuse have selected for nosocomial isolates presenting intrinsic and acquired multidrug resistance determinants. It has been considered that resistance against carbapenem is, in itself, sufficient to define an *A. baumannii* as highly resistant. The molecular basis of carbapenem resistance in this species has been ascribed to the recruitment and production of carbapenem-hydrolyzing class D β-lactamases (CHDLs) and, to a lesser extent, of metallo-

β-lactamases (MBLs). In A. baumannii, the CHDLs can be intrinsic (OXA-51-like) or acquired (OXA-23-like, OXA-24-like and OXA-58-like). Although these enzymes weakly hydrolyze carbapenem, they can confer high resistance when blaOXA genes are overexpressed, as a result of their association with mobile elements, such as ISAba1, which carries a strong promoter [9]. In addition to this enzymatic resistance,Porins are outer membrane proteins (OMPs) able to form channels allowing the transport of molecules across lipid bilayer membranes. Variations in their structure or regulation of porin expression can provide a mechanism to escape from antibacterial pressure.[32] *A. baumannii*intrinsically have a smaller number and size of porins compared with other Gram negative organisms, contributing to the intrinsic outer membrane impermeability.The loss of membrane permeability, due to alterations in specific porins, is an intrinsic carbapenem resistance mechanism in *A. baumannii*. The 25/29 kDa heat-modifiable carbapenem-associated outer membrane protein (CarO) is a porin with a β-barrel topology. This channel allows the selective uptake of amino acids and Imipenem due to its structural conformation and to the presence of an Imipenem binding site. This site was not found in any other OMP, and thus, CarO protein is considered a preferred uptake channel for this antibiotic. In this way, *carO* gene alterations, as disruption by insertion sequences, changes in the primary structure mainly in the Imipenem binding site or decreased expression, would have a dramatic impact on the entry of Imipenem in the cell, thus contributing to resistance to this antibiotic [33].

Plasmid curing

Curing of plasmid in A. baumannii isolate A33 by Ethidium bromide

Ethidium bromide had been used at concentration (50,75,100,125,150,and 175µg/ml) to investigate the curing action against plasmid DNA content which showed resistance to most antibiotics understudy in A33isolate.*Table: 2*demonstrate the curing percent of plasmid DNA from *A. baumannii*A33 by ethidium bromide in which (125µg/ml) was the most effective concentration for this process.For A33 isolate, ethidium bromide affect ceftriaxone, Vancomycin, penicillin,and doxycycline genes, the resistance rate of A33 isolate decreased and the percent of curing reached (36.3%).

Table- 2: Curing of plasmid DNA from *A.baumannii*isolate A33 isolate by using Ethidiumbromide (EtBr)

Treatment	Antibiotics											
	IMP	CRO	GM	CEFA	VA	P	E	S	CTX	DOXY	CIP	Percentage
A33	S	R	R	S	R	R	S	R	R	R	S	36.3%
EtBr +A33	S	S	R	S	S	S	S	R	R	S	S	

Figure: 3 shows the plasmid DNA profile for A. baumanniiisolate (A33) that contain two bands before curing and it is clear that one plasmid DNA was remain in the A33 isolate after treating with ethidium bromide .

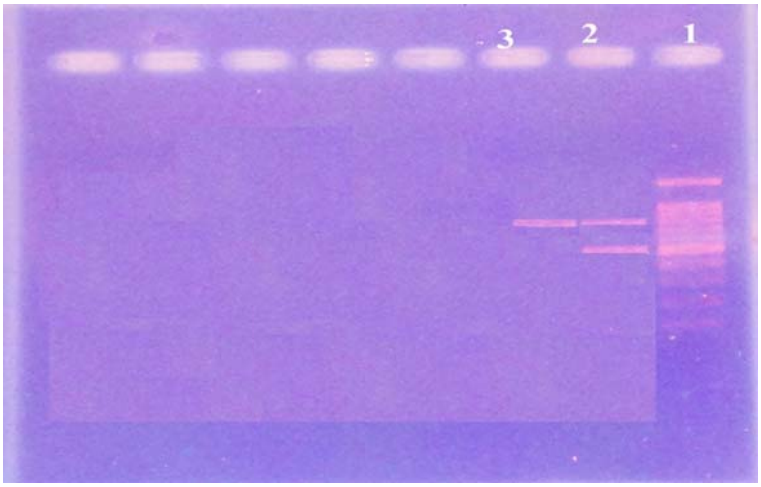

Figure- 3: Plasmid profile of *A. baumannii* before and after curing

Among various plasmids present in bacterial strains, R plasmids are very significant as they confer resistance to one or more antibiotics thus possess a threat to chemotherapy. Treatment of cells with certain chemical and physical agents enhances the elimination of plasmids from host cells. This phenomenon is referred to as curing and has been used to ascertain the plasmid associated nature of genes. Susceptibility to curing agent also varies among plasmids [34].Our results agreed with [35] who used five isolates of *A. baumannii* that showing resistance to all antibiotics for plasmid curing. The results showed that *A. baumannii*was cured (with 60% frequency) using ethidium bromide as curing agent, so it can be interpreted that probably antibiotic resistance markers of these bacteria are plasmid mediated. [36] mention that curing of plasmids by ethidium bromide at a subinhibitory concentration of 512 μg/ml was highly efficient. The disappearance of antibiotic resistance to expanded-spectrum cephalosporins with the concurrent loss of all three plasmids from all *A. baumannii* isolates suggested that the ESBL determinants were plasmid-borne.[7] studied *A. baumannii*isolates with multiple plasmids isolated from hospital for their curing by ethidium bromide as chemical agent, they found that these R plasmids are incurable by acridine orange but were successfully cured by treatment with ethidium bromide. [37] used ethidium bromide for curing experiment of three isolates of *P. mirabilis* that bearing plasmids for detecting the location of antibiotic markers on plasmid or on the chromosomal DNA. As a result, they lost their plasmids and resistant to β-lactam antibiotics as Ampicillin, Amoxicillin, Cephalothin, Cefotaxime, Ceftriaxone, Tetracycline and Gentamycin. The effect of ethidium bromide as curing agent might be due to their action as intercalating agent. This mutagen became inserted between two base pair can lead to insertion or deletion, thus can induce frameshift mutation. On the other hand, the unsuccessful of ethidium bromide in curing process for certain antibiotic genes may be attributed to their failure in inhibition of plasmid replication that carry resistance to these antibiotics due to incapability to create mutation in the original replication that specific for this plasmid, or because of their disability to prevent plasmid distribution mechanism on daughter cell as a result remaining of these plasmids in these isolates [38].

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