Sawsan Mohammed Kareem et al /J. Pharm. Sci. & Res. Vol. 10(6), 2018, 1319-1322

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Genetic Detection of *Acinetobacter* Multidrug Resistant isolates toward Aminoglycoside and Study the Resistant to Rifampicin/Aminoglycoside Combination

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Abstract

Forty five samples of urine were collected from patient with UTI in medical city, in Baghdad. At first all sample were cultured differentiation media (MacConkey & Nutrient agar) under suitable conditions, (20) isolates have been identified as *Acinetobacter*.

Agar diffusion method was used to five antimicrobial agent toward all *Acinetobacter* isolates, the isolates showed different sensitivity against antimicrobial agents used. the isolates appeared resistance to more than one antimicrobial agents. The isolates display sensitivity toward aminoglycoside group (Amikacin AK 60% & Gentamycin 30%), While moderate sensitivity toward quinolones group (Ofloxacin 30% & Levofloxacin 20%) and finally which was observed Rifampicin the lowest effective antibiotic toward these isolates the sensitivity was (10%) toward *Acinetobacter* isolates.

Agar dilution method was applied to determination of minimum inhibitory concentration (MIC) to antimicrobial agents (Rifampicin, Gentamicin and Amikacin) each of them separately, furthermore applied combination of (AK & R) and (CN & R), the results showed all *Acinetobacter* isolates were resist to rifampicin (100%), Gentamicin (80%) and Amikacin (60%) alone in all concentration of MICs. While results of combination of antimicrobial agents showed increase the sensitivity toward them, the synergic effect of the combination between (AK & R) up to (80%) while the combination between (CN & R) up to (60%).

Polymerase chain reaction PCR was used to screening about some aminoglycoside modifying genes prevalence in all resistant isolates, the results showed predominance of (ant (4)IIb 12.5%, aac (6)Ib 12.5%, aph (3)VI 56.25% and 18.75% of isolates showed negative results to these genes.

Keywards: *Acinetobacter*, rifampicin, aminoglycoside genes, MDR bacteria

INTRODUCTION

Acinetobacter baumannii prior to the 1970s, a troublesome opportunist surreptitiously gained recognition as a significant pathogen. It include heterogeneous collection of gram (–ve) coccobacillus, strictly aerobic, non-fermentative, non-motile, no spore forming, (-ve) oxidase and (+ve) catalase. It found commonly in pairs, but also maybe found in chains of variable length. The flagella was absent, despite of it conceder non-motile, the "twitching" or "gliding" motility was recorded to occur in semisolid media [1]. The pathogenicity basically depended on cell surface component and hydrolytic enzymes. These two virulence factors play important role in antimicrobial resistance of Acinetobacter spp. isolates. The virulence factors that documented include novel pilus assembly system participated in biofilm formation [2], and an outer membrane protein (Omp38) which play role in stimulate apoptosis process in human epithelial cell [3].

The big challenge with *A. baumannii* isolates, it has ability to acquired rapidly antimicrobial resistance genes causing to multidrug resistance MDR, the misused of antimicrobial within hospitals leading to emergence and increasing MDR among *Acinetobacter ssp.* specially, vast used of extended –spectrum cephalosporins and quinolons [4, 5]. Furthermore MDR *Acinetobacter baumannii* is a decidedly formidable pathogen and causes nosocomial infections belong to high morbidity and mortality at hospitals [6,7]. Biofilm formation play role in enhancing the pathogenicity and resistance initially on clinically important surfaces [8]. Adhesion and subsequent formation of biofilm due to vast number of genetic factors in MDR *A. baumannii* which are till now not completely clear [9]. Antimicrobial agent Aminoglycoside group was attack the bacteria by process with two steps, firstly uptake of Aminoglycosides inside bacterial cell which considered the important process for their biological activity. Secondly, Aminoglycoside will bind with small subunit 30s of ribosome in the bacterial cell will leading to inhibit protein synthesis and misreading of mRNA causing dysfunctional protein production [10,11].

Many antimicrobial agent such as rifampicin, sulbactam, imipenem and colistin were used to treatment *A. baumannii* [12]. Many studies have been demonstrated that the combination of antimicrobial agents in vitro such as rifampicin & colistin, imipenem & colistin and rifampicin & imipenem leading to increase butter activity against MDR *A. bumannii* [13]. Rifampicin has capacity to inhibit transcription process due to their binding with bacterial DNA-dependent RNA polymerase despite of the well characterization of molecular target of rifampicin the precise mechanism still unclear [14]. The combination between antimicrobial agents seems to be an effective alternative way in treatment *A. baumannii.* The main mechanism of resistance in *A.bumannii* to aminoglycoside antibiotics representative by alteration of hydroxyl or amino group in aminoglycosides, the alteration causes reduce or decrease binding of antibiotic with ribosome, a lot of previous studies had been indicted that there many resistance techniques in *Acinetobacter ssp*. toward aminoglycoside [15]. The generally prevailing of resistance mechanism is belong to enzymatic inactivation by acetyltransferases (AACs) which encoded by (*acc*) gene, phosphotransferases (APHs) which encoded by (*aph*) gene and nucleotidyl transferases (ANTs) which encoded by (*ant*) genes, although the numerous of the aminoglycoside modification enzymes leading to clinical resistance however AACs and APHs responsible for high level of resistance [15,16].

So this study aimed to define and compere between the in vitro activity of aminoglycoside and rifampicin separately and the combinations of these antibiotics, furthermore screening about some aminoglycoside alteration enzymes genes in *A. baumannii* isolates which isolated from Baghdad Hospitals.

MATERIALS AND METHODS

Collection of samples & Bacterial identification

Forty five samples of urine were collected from patient suffering from UTI in medical city Baghdad hospitals. Then all the samples were cultured on different cultural media (Brain heart infusion agar, MacConkey agar and blood agar after incubated at 37°C/24 hrs. the identification of *A. baumannii* was done by taken single colony form primary (+ve) culture, depending on morphological properties of colonies furthermore the isolates subjected to Gram stain and biochemical test to observed reactions according to diagnostic procedures MacFaddin (2000).

Antibiotic sensitivity Testing

Sensitivity of bacterial isolates was carried by disc diffusion methods according to guidelines of (CLSI, 2013). Antibiotic discs were placed on Muller –Hinton agar plates, incubated at 37oC/ 18 hrs. the diameter of each inhibition zone was measured, the antibiotic were used: Gentamicin CN 10 µg, Amikacin AK 10µg, Rifampicin RA 5 µg, Ofloxacin 5µg, Levofloxacin 5µg.

Determination of Minimum Inhibitory Concentration (MIC)

Stock solution with final concentration 10mg/ml of antibiotics Gentamycin , Amikacin and Rifampicin were prepared according to CLSI [16] recommendations by using distilled water, the sterilized by filtration and storied at -20^{o}C in small containers, MIC test was done to all isolates by applies standard agar dilution method as recommended in CLSI [17], The serial two fold dilution of each antibiotic ranging from 0.5 to 1024 µg/ml. Gentamycin , Amikacin and Rifampicin were used with Muller-Hinton agar medium which prepared by autoclave and leaved to cooled at 45^{o}C then the antibiotics were added in appropriate amount from their stock solution , mixed well and poured into the plates. Transfer few colonies from overnight bacterial culture to 2 ml of normal saline to prepare 1.5×10^{8} CFU/ml. 5µl of bacterial suspension was placed on agar surface by micropipette, then leave to dry and incubated at 37^{o}C/18hrs. MIC results were read after 18-24 hrs. And MIC recorded at the lowest concentration from antibiotic that inhibit bacterial growth.

Antimicrobial combination testing

The antimicrobial combination (Combination of Gentamycin and Rifampicin, Amikacin and Rifampicin) testing was carried out via prepare serial dilution of antibiotics, which required in test and get the require concentration which if we take 0.1ml (100µl) from it this volume will be carried the same concentration which we needed. Cultured MHA plates with required bacteria by using lawn streaking. Making wells in MHA plates by using cork borer and loaded by require concentration. Incubate the plates at 37^{o}C for 24 hours, and read the results. [18].

Genomic DNA extraction and PCR reaction

Sixteen resistant isolates of *A. bumannii* to aminoglycoside antibiotics were cultured on MacConkey agar for 18 hour at 37^{o} C and then the isolates were collected and suspended in normal saline, DNA was extracted from suspension by (WizPrep^{TM} gDNA mini kit-cell/tissue) according to the leaflet of manufacture company (wizbiosolution/ Korea), then the purity of DNA was measured by Nano drop, finally eluted in water and kept at -20^{o}C pending used. Detection about resistance gene was carried by Multiplex PCR using specific primers to aminoglycosides modifying enzymes genes (aph(3)VI, ant(4)IIb and aac(6)Ib).

The sequence of oligonucleotide PCR primers were used in this study listed in table (1), PCR reaction mixture was carried according to the procedure was mentioned in the leaflet of manufacture company (promega/USA) that refer to 3µl of DNA template, 1µl (0.6 pmol) of each primer F&R, 12.5 µl of 2X GO Taq®Green mastermix and finally completed the volume by adding nuclease free water to 25µl. the conditions of amplification steps have been done by using thermocycler (TechNet-500/USA), the initial denaturation 95C/5 min., then initial denaturation 95^{o}C/30 sec., annealing 55^{o}C/30 sec., extension 72^{o}C/60 sec. repeated to 35 cycles after final extension at 72^{o}C/ 10 min. the PCR products were subjected to electrophoresis by using 1% agarose stained with ethidium bromide 0.5 µg/ml at 7 V/Cm /90min and DNA ladder (100bp promega/USA) lastly the gel visualized under UV trasilluminator.

RESULTS AND DISCUSSION

Collection and diagnosis of the isolates

The results of cultured forty five urine samples on different cultured media were showed twenty of these cultured positively confirmable as *Acinetobacter* followed by epi and chemical diagnostic tests .

Antimicrobial susceptibility test for *Acinetobacter*

Antimicrobial susceptibility test of all the isolates to different antibiotics was determined, the results showed different susceptibility against antibiotics used with resistance to more than one antibiotics MDR. The isolates sensitivity to aminoglycoside group Amikacin and gentamicin 60%, 30% consequence while the sensitivity toward quinolones group Ofloxacin and Levofloxacin 30%, 20% consequence furthermore the sensitivity toward rifampicin was the lowest only 10%. These results compatible with [20] that show the percentage of sensitivity toward amikacin (45%), gentamicin (47.5%) and Rifampicin was found the less effective antimicrobial agent toward *Acinetobacter baumnnii* isolates. as shown in Figure (1)
Only four isolates in our study were MDR to all antibiotics used. *Acinetobacter* the most difficult to treat because of selective resistance to previously effective combination drugs. Studies of imipenem and meropenem (carbapenems) combined with aminoglycosides have proven effective in some cases, although outbreaks in Spain of carbapenem resistance (as high as 80 percent) as reported by [21].

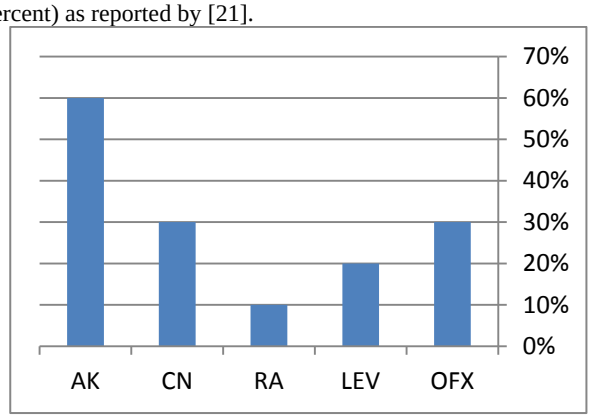

Figure 1: Antimicrobial susceptibility test for Acinetobacter

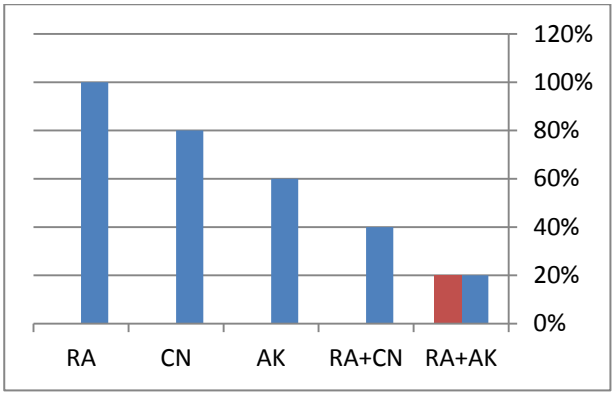

Figure 2: MIC test and combination between antibiotics test

MIC and combination between antibiotics test

The determination of the Minimum inhibitory concentrations for Rifampicin, Gentamicin and Amikacin was done to all isolates in disc diffusion methods as a complementary test to determine the break point, the resistant isolate characterized by CLSI if MIC greater than break point while it susceptible if less than break point.

Table 1: The sequences of oligonucleotide primers was used in this study

Gene	Sequence of oligonucleotide 5`----------3`	Product size (bp)	Reference
aac(6)Ib	F- TTG CGA TGC TCT ATG AGT GGC TA R- CTC GAA TGC CTG GCG TGT TT	482	19
ant(4)IIb	F- GACGACGACAAGGATATGGAATTGCCCAATATTATT R- GGAACAAGACCCGTTCAATTCAATTCATCAAGTTT	364	19
aph(3)VI	F- TAT CTC GGC GGC GGT CGA GT R- CAC GCG GGG AAA CGC GAG AA	800	19

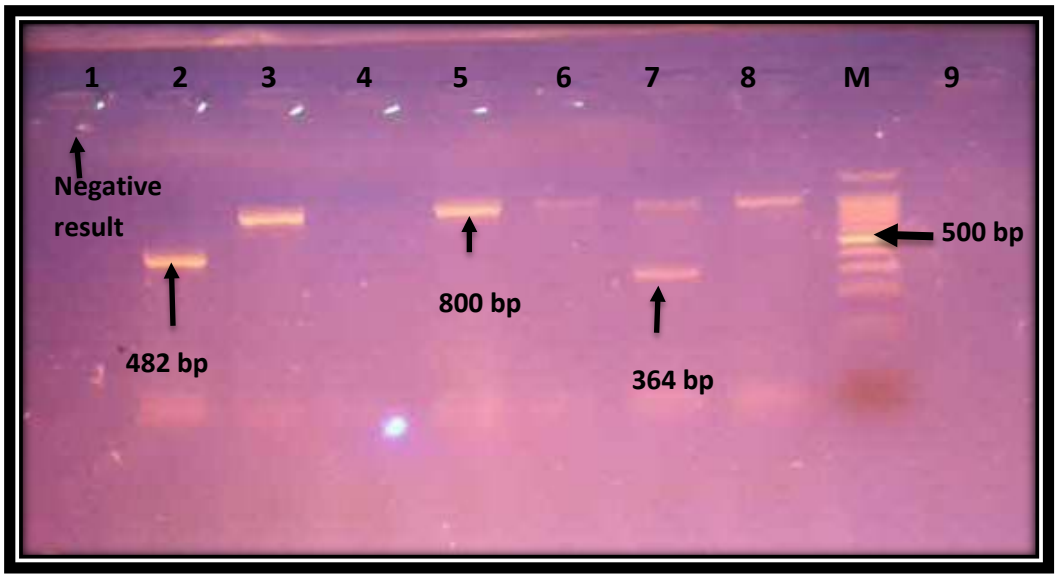

Figure 3: amplification of *ant(4)IIb* (364bp), *aac(6)Ib* (482bp) and *aph(3)VI* (800 bp) genes by Multiplex PCR; line M DNA ladder (100 bp)

MIC result showed Acinetobacter isolates resist to rifampicin 100%, Gentamycin 80% and Amikacin 60% separately, this result compatible with [22] that referred to both strain resist to rifampicin at MIC/MBC 8/8 mg/L. On the other hand our results showing decreased in resistance by synergic effect to combination between rifampicin & gentamycin 40% and rifampicin & amikacin 20%. Our results agreement with [23] that show rifampicin may be useful to treatment MDR *Acinetobacter baumannii* isolates in combination with other drugs such as polymyxins, sulbactam, and carbapenems. In vitro synergy is frequently observed between polymyxins and rifampicin.

Aminoglycoside modifying enzymes (AME)

The genotypic detection techniques play important role in rapid and sensitive detection methods to determine resistance genes furthermore become more accessible in clinical microbiology laboratories and appear as alternative to the classical identification and detection protocols [24]. The screening of aminoglycosides modifying enzymes genes by PCR technique have been revealed that predominant of *aac(6)Ib* gene in two isolates 12.5 %, the aminoglycoside acetyltransferase enzyme responsible for amikacin and gentamicin resistance while the prevalence of *ant(4)IIb* gene in two isolates 12.5 %, the aminoglycoside adenylyl transferase enzyme confer the resistance toward amikacin, tobramycin and isepamicin [25]. Finally the major prevalence gene was *aph(3)VI* gene, that appear in nine isolates 56.25 % as shown in figure (3). three resistant isolates 18.75% had no AME genes. Several previous studies have examined the occurrence of aminoglycoside modification enzymes genes in *A. bumannii* Iraqi isolates [18] showed that prevalence of *aac(6)Ib* gene 46.66%, *aph(3)VI* gene 13.33% and *ant(4)IIb* gene 33.33% also [26] reported prevalence of AME *aac(6)Ib* gene 83.6%, *aph(3) VI* gene1.6%. on the other hand, we detected two isolates harbored with two AME genes *ant(4)IIb* and *aph(3)VI* simultaneously.in fact the spreading the AME genes belong to location of these genes on mobile genetic elements such as plasmid, transposon or integrons which facilitate transmission between bacteria [27].

Conclusion

A.baumannii a powerful nosocomial pathogen that consider high risky pathogen to immunocompromised and hospitalized patient so the results of this study encourage used combination between rifampicin/ amikacin or rifampicin/ gentamycin to treatment MDR *Acinetobacter baumannii* infections. Furthermore using of rifampicin with other combinations should be explored specially antibiotic has a good activity in vitro. We encourage further explore about the combination between antibiotics in vitro and in vivo due to butter results belong to combinations than using alone to Gentamicin or Amikacin to prevent develop high resistance to antibiotics. Moreover the detection of resistance genes in resistant isolates play important role to control and manage the treatment also discover new trends to limit spreading this pathogen in the world.

Acknowledgment

We would like to express our thankful to biology department/ college of Science / Mustansiriyah University (www.uomustansiriyah.edu.iq) for facilitating and supporting our work in this research.

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