Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 1581 - 1591
Received 16 February 2021; Accepted 08 March 2021.

Detection of Colistin Resistance Genes in *Acinetobacter Baumannii*isolated from Different Clinical Specimens

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Abstract:

The antimicrobial resistance are a worldwide increasing problem. Colistin represent a major group of polymyxins class that mostly resistance by gram negative bacteria, so the detection of colistin resistance genes are very important fo`r optimal patients care. Colistin (polymyxin E) old antibiotic used to treat infections caused by multiple drug resistant (MDR) *Acinetobacter baumannii* isolates. The aim of this study is to detect the colistin resistance in *A. baumannii* isolates obtained from patients with various clinical specimens. Six hundred specimens were collected during the period from September 2020 to December 2020 from three hospitals in Babylon Province/ Iraq. The clinical specimens included burn, blood, wound and urine. Morphological and biochemical tests were used for isolation and identification of bacterial isolates. These isolates were obtained as a pure and predominant growth from clinical specimens. Antibiotic susceptibility tests were carried out using disk diffusion method (DDT) and MIC by agar dilution method for phenotypic detection of colistin resistant isolates. PCR technique was performed to detect Mobilized Colistin Resistance (MCR) genes. Twenty isolates of *A. baumannii* out of 600 specimens (3.33%) were detected. Moreover, 9 isolates out of 20 tested(45%) showed heteroresistance to colistin by DDT while Minimum inhibitory concentration by agar dilution method showed 3(15%) out of 20 *A. baumannii* isolates resistant to colistin and have MIC value, 128 µg/ml. Furthermore, Colistin resistance genes (*mcr1, mcr 2, mcr3, mcr4, mcr5*) were investigated by PCR as following: *mcr4, mcr5* detected predominantly in 15(75%),11(55%) out of 20 *A. baumannii* isolates respectivel, however *mcr1*gene was detecetd in 3 (15%). While *mcr 2, mcr3* were not detected in this study. This study included that, there was emergence of colistin resistance in MDR *A.baumannii* and might hamper the efficacy of colistin as alternative therapy in these isolates.

Keywords:*Acinetobacter baumannii*, Susceptibility testing, MCR, Colistin resistance genes.

Introduction:

Acinetobacterbaumannii is gram-negative bacteria, non-motile, bacillus, pleomorphic and aerobic and opportunistic bacteria. It occurs and spread among immunocompromised patients, especially those who stay in the hospitals for a long time. It usually colonizes the skin, respiratory and oropharynx. It is considered red alert bacteria because it has wide antibiotic resistance. Antibiotic resistance is a worldwide problem, with numerous bacterial infections having already become very challenging to treat .Colistin is often used as last antibiotic to treat infection of

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Received 16 February 2021; Accepted 08 March 2021.

multiple resistant bacteria, However, an increasing amount of bacteria previously susceptible to colistin have been showing resistance against it, this is a problem that threatens humanity[1].

Colistin belongs to the antimicrobial class designated polymyxins which originates from the organism *bacillus polymyxa*, colistin (polymyxin E) used in clinical practice .It is one of the few last-line antibiotics used in treating deadly infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacilli GNB. Colistin acts by targeting the lipopolysaccharide component (LPS) of the outer membrane, with the lipid A component of this membrane. The interaction between the phosphate groups of lipid A and the acid residues of colistin displaces the divalent cations: magnesium (Mg2+) and calcium (Ca2+).This renders the cell destabilized and vulnerable. However, after the increasing prevalence of multi-drug resistant bacteria by the mid-1990s, it was once again applied in the treatment of infections. Two forms of colistin are administered to humans: colistin sulphate and colistin methanesulphate (CMS). Colistin sulphate is commonly used for either selective digestive tract decontamination or the treatment of skin infections. CMS is administered as a last resort drug against bacteria with resistance against multiple other antibiotics. Most commonly, it is used in case of an infection by Gram negative multiple-resistant bacteria like *Pseudomonas aeruginosa*, *Acinetobacte*r *baumannii* and *Klebsiella pneumonia* or *Enterobacter* spp [2].

The Plasmid-mediated colistin resistance due to the mobilized colistin resistancegenes(MCR)genes,poses a big threat as it has the capacity to spread across the world at a very fast rate. These*mcr*genes has already been found on many types of plasmids with various sizes, which indicates its capacity to rapidly spread amongst bacteria.Since the first discovery in 2015, 10 different *mcr* genes have been identified worldwide .However, recent studies have revealed a global spread of mobile colistin-resistance (*mcr*) genes, which are genetic elements that encode colistin resistance, raising public health concerns. These genes affect colistin efficacy and can restrict treatment options for complicated infections. Despite widespread interest, many aspects of the molecular epidemiology of *mcr* remain poorly understood. Treatment of infections caused by multidrug-resistant bacteria, such as those caused by *Acinetobacter baumannii*, is a real challengedue to a lack of effective antibiotics, colistin, an old antibiotic, has recently been used as a last therapeutic alternative. It has been reclassified as an antibiotic of vital significance in human clinical settings.The aim of the research was to characterize colistin resistance in *A. baumannii* isolates obtained from patients with various clinical hospital infections, both phenotypically and genotypically[3].

Materials and Methods

1- Isolation and Identification of *A. baumannii* Isolates:

Different clinical specimens (burn, wound,urine and blood) were collected from patients attended hospitals (Al-Hilla Teaching Hospital ,Medical city of Mirjan and Imam Al-Sadiq Hospital in Babylon Province/ Iraq). During the period from September 2020 toDecember 2020. The spesimens were immediately inoculated onMacConkey agar and then on CHROM agar incubatedfor overnight at 37°C under

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aerobic conditions. The bacterial isolates were obtained as a pure and predominant growth from clinical specimens and identified according to [4,5].

2- Antibiotic Susceptibilty profile:

The antibiotic susceptibility profile for 20*A. baumannii* isolates was determined using disk diffusion test (DDT) and interpreted as recommended by the CLSI,2020.The antibiotics used in this study were carbenicillin (100µg), cefepime (30µg), cefotaxime (30µg), imipenem (10µg), amikacin (30µg), tetracycline (30µg) and ciprofloxacin (5µg)**.**However,DDT was used for detection heteroresistance of colistin in *Acinetobacter baumannii* isolates as recommended by [6,7].Heteroresistance phenomena ,defines by the presence of different sub-populations within an isolate that exhibit varying susceptibilities towards an antimicrobial agentthat appears in DDT only. Furthermore, minimum inhibitory concentration (MIC) by agar dilution method as recommended by[8].

3-Molecular detection of ColistinResistance genes (*mcr*) by PCR:

Genomic DNA of bacterium was extracted according to the genomic DNA purification kits(G-spin) supplemented by manufactured company(Bioneer,Korea). Each 25µl of PCR reaction mixture contained 0.5 µl of upstreamprimer, 0.5 µl of downstreamprimer, 11.5µl of nuclease freewater, 1µl of DNA extraction and11.5 µl of master mix. The PCR product of 5 primers (*mcr1, mcr 2, mcr3,mcr4, mcr5*) The Primer sequences and thermal cycler conditionare listed in Table (1).The amplification productswere separated in 1%agarose. DNA ladder (100-1500bp) (Bionear, Korea). After electrophoresis, thegel was photograhed under UV light.

Table (1): Primer sequences and thermal cycler condition

Genes	Primer sequence (5'-3')	PCR-condition	Number of cycles	References
mcr-1	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	95°C/5m 95°C /30s 55°C /30s 72°C /1m 72°C /5m	35	*(*Rebelo*etal., 2018))*
mcr-2	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC	95°C /5m 95°C /30s 53°C /30s 72°C /1m 72°C /5m	35	
mcr-3	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	95°C /5m 95°C /30s 47,48,50°C /30s 72°C/1m 72°C /5m	35	

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mcr-4	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG	95°C /5m 95°C /30s 52°C /30s 72°C /1m 72°C /5m	35	
mcr-5	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	95°C/5m 95°C /30s 51°C /30s 72°C /1m 72°C /5m	35	(Borowiak *et al.*, 2017)

RESULT AND DISCUSSION

solation and identification of Bacterial Isolates

The results of this study showed that among 600 clinical specimens, 20(3.33%) isolates were identified as *A.baumannii*.Theseresults were slightlymore than the results of other local study[9], who found that the isolation rate (2.40%), despite that [10], found that the isolation rate of *A. baumannii* was (13%).Other study that conducted by [11], revealed that the isolation rate of *A. baumannii* was (9.51%).Furthermore, [12],presented*A.baumannii*as(55.6%). The disparity in the isolation rate in whole studies may be due to numerous factors such as the differences in geographical distribution and seasonal variations.Many infections of the *Acinetobacter* have a seasonal variation, it has been defined that *A. baumannii* usually grows in moist environments, more humid ambient air,favoring *Acinetobacter* infection , Numerous outbreaks have been traced to liquid or wet environmental reservoirs that have promoted the dissemination of *Acinetobacter* species, Furthermore, the frequency of community-acquired *A. baumannii* infections has been increasing gradually.

2-Antibiotic Susceptibility Profile of *A.baumannii* isolates:

The susceptibility of 20 isolates of *A. baumannii* towards 7 different classes of antibiotic were evaluated by using DDT.The evaluation and interpretation of results were done according toCLSI,2020.All isolates(20) were resistant to β- lactam, with rate resistance (100%), which was simillar to study from different hospital in Thailand[13], which found that *A.baumannii* isolates were resistance 100% for Imipenem , while other study [14], showed resistance rate (66.6%)for imipenem. While the third generation cephalosporins includingcefotaxime, showed resistance rate (100%) which was simillar to local study by[15], who found resistance 100% for Cefotaxime, however,[16], found the resistance rate, (80%).Also fourth-generation cephalosporins , Cefepime also showed high resistance rate (100%) which was similar to studyof[17], who found that *A.baumannii* isolates were resistant 100% for Cefepime, Furthermore in this study , high resistance rate *A .baumannii* for Amikacin, tetracycline (100%) for each , Which was similar to the results that conducted by [14],while in study[18],showedresistance rate (60%).The resistance to Fluoroquinolones, Ciprofloxacin, also showed high resistance rate (100%), which was more than the result of [19], who found (76 %), resistance ratefor Ciprofloxacin.In the current study, all bacterial isolates wereMDR as resistant to at

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least one agent in ≥3 antimicrobial agents class[20], The appearance of MDR *A. bumannii*isolates has caused many problems in the treatment of these isolates.In this study, all classes of antibiotics listed in CLSI,2020 were used therefore consider MDR and XDR. In the present study and other studies the variation in resistance ratios may be due to the diversity of isolated sources and to the acquisition of resistance genes.In this study 9 out of 20 isolates of *A.baumannii* showed rate of heteroresistance(45%) , this was relatively similar to the study of [21], who showed 33% colistin heteroresistance in *A. baumannii* isolates.Several other studies were also identified by[22],detected heteroresistance rates vary from 18.7 to 100%.On the other hand,[23], reported9 colistin heteroresistance out of 576 *A.baumanni*i isolates.This is the first study in Babylon and in Iraq, focusing on Heteroresistance of colistin in *A. baumannii* isolates. Despite, CLSI (recommended MIC for detection colistin resistance as used in the current study also to evaluate the resistance and susceptibility phenotype in *A.baumannii* isolates of the current study.

Agar dilution method was used in the current study to evaluate the colistin resistance in *A. baumannii* by measurement of minimum inhibitory concentrations (MIC) according to the CLSI, 2020 recommendations.The results showed colistin resistanc in 3 isolates out of 20 (15%)in a concentration of (128µg /ml) while the rate of susceptiblity, is 17 (85%) in *A.baumannii* isolates with MIC range (0.125-64) µg /ml according to the CLSI,2020.These findings were comparable with the study of[24], who recorded the MIC value (64-128 µg /ml), resistance for colistin. In Egypt, it was first reported by[25], in 5% (2/40) isolates (have MIC values 4 and 8 mg/ml) by agar dilution. However,[26], recorded(31.8 %)*A. baumannii* isolatesresistantto colistin detected by agar dilution method with an MIC value 32 g/ml.

The Agar dilution method was used for the detection of colistin resistance to evaluate the emergence of resistance in *A. baumannii* clinical isolates as it has been reported worldwide. This method, confirms not only the equal distribution of colistin in the agar plates and reliability of this method for MIC determination but also a good stability of antibiotic in MH agar under the proper storage conditions. Agar delution has been shown to be reliable for colistin MIC determination in several studies [27], The agar dilution method was found to be superior in terms of reproducibility.

3-Molecular characterization of Mobilized Colistin resistance(MCR) genes (*mcr1, mcr2, mcr3,mcr4, mcr5*) by PCR:

The conventional PCR was used in the current study for characterization of *mcr* genes,fragments of five *mcr* genes (*mcr-1* to*mcr-5*) represented amplicon size ranged from 320 bp (*mcr-1*) to 1644bp (*mcr-5*) (Fig.1,2,3,4,5). The detection rates were as following; *mcr-1* gene in 3 isolates out of 20 (15%), while,*mcr-4* and *mcr-5* genes showed 15(75%),11(55%) out of 20 *A. baumannii* isolates respectively. However, *mcr-2* and *mcr-3* genes showed negative results. Comperable with other study [1], Whofound *mcr-4* gene10(76.9%) followed by *mcr-1* gene 5 (38.5%), 4(30.8%) for both*mcr-2* and *mcr*-3genes respectively. While no*mcr-5* gene was found in *A. baumannii* isolates. However,[28], showed thatthe *mcr-1* gene was detected in *A. baumannii* (16.6%) which was comparable with the (15%) of current study.

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In other previous studies, the prevalence of *mcr-1* has only been investigated in *E. coli* and *K. pneumoniae*[29]. But inthe present study,*mcr-1* genes in *A.baumannii*also determined. The emergence of colistin-resistance in *A. baumannii* isolates were alarming because it further limits therapy options and requires prudent antimicrobial stewardship and stringent infection control measures.

Mobilized colistin resistance (*mcr*) genes has been observed in different countries with different frequency in various specimens.Since colistin is often considered the antibiotic of last resort for treating MDR in Gram-negative pathogens, the rise in colistin resistance is a cause for concern. It is crucial to understand the mechanisms of colistin resistance, not just for surveillance but also to find new targets for antimicrobial drug production[30].

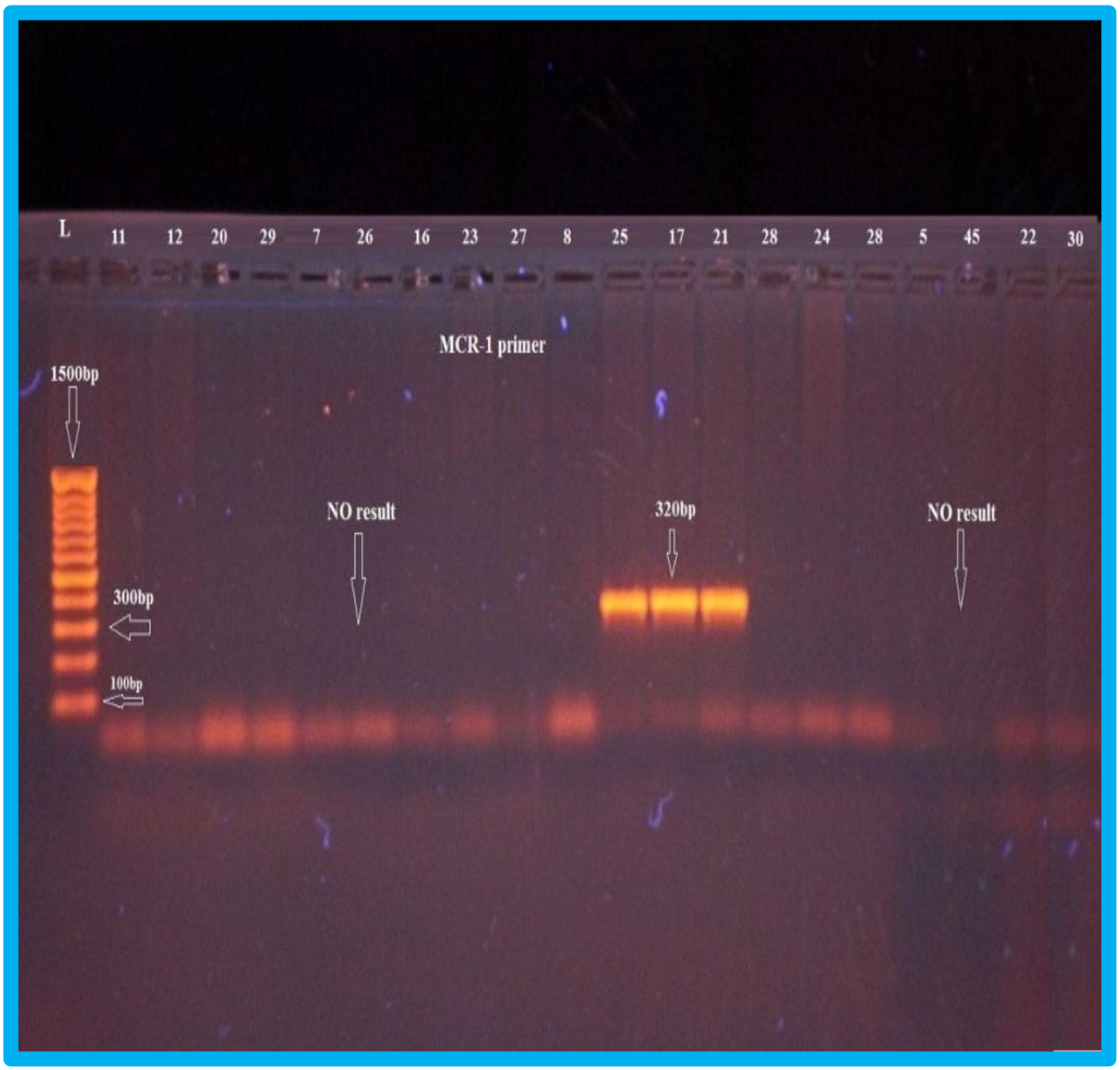

Figure (1) :- Gel electrophoresis for PCR product of (*mcr-1* primer) showed 320 bp (Agarose 1%, 10min. at 100 voltage and then lowered to 70 volts, 60min.).Visualized under U.V light after staining with ethidium bromide. Lane L : DNA ladder (100-1500bp), Lanes (25,17 and 21)

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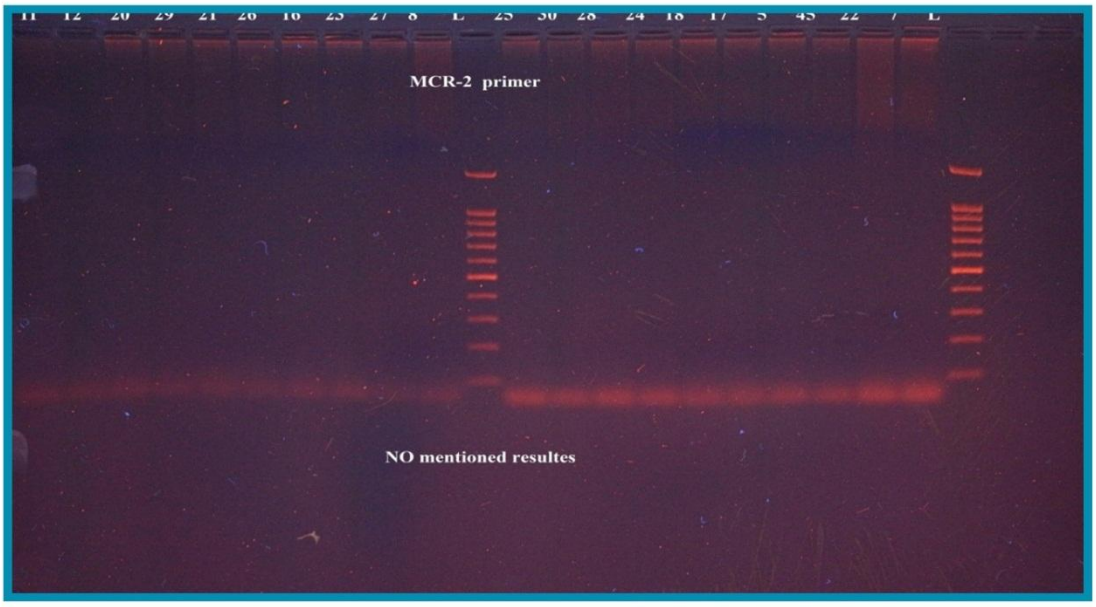
represented positive results of*bacterial DNA* isolates, Lanes(11-8 and 28 - 30) represented Negative results lane N represent negative control .



Figure(2) :- Gel electrophoresis for PCR product of (mcr-2 primer). (Agarose 1%, 10min. at 100 voltage and then lowered to 70 volts, 60min.).Visualized under U.V light after staining with ethidium bromide. Lane L : DNA ladder (100-1500bp), Lanes 11– 7: represented negative results of bacterial DNA isolates.

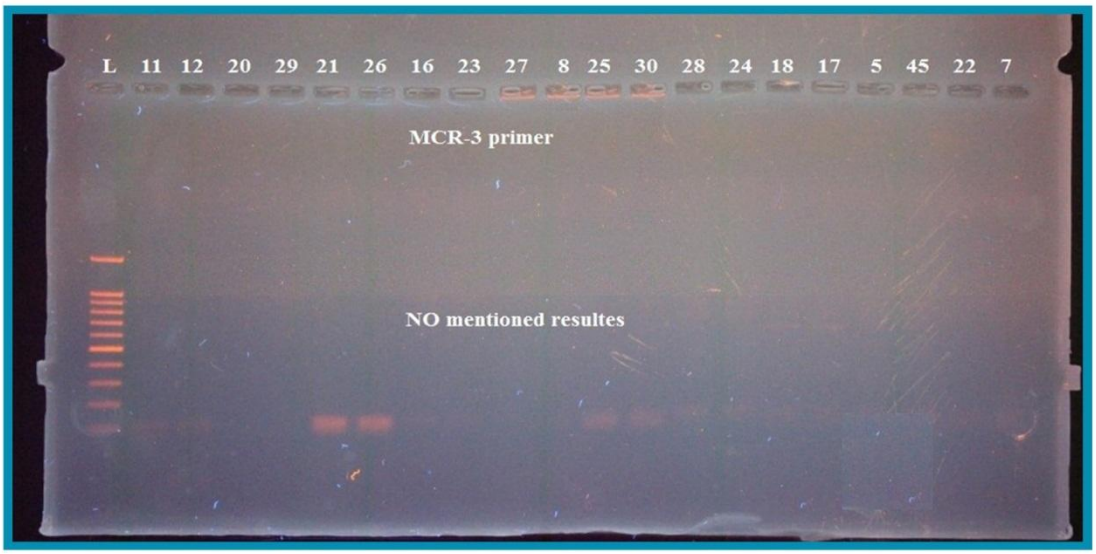


Figure (3) :- Gel electrophoresis for PCR product of (mcr-3 primer). (Agarose 1%, 10min. at 100 voltage and then lowered to 70 volts, 60min.).Visualized under U.V light after staining with ethidium bromide. Lane L : DNA ladder (100-1500bp), Lanes 11– 7: represented negative results of bacterial DNA isolates.

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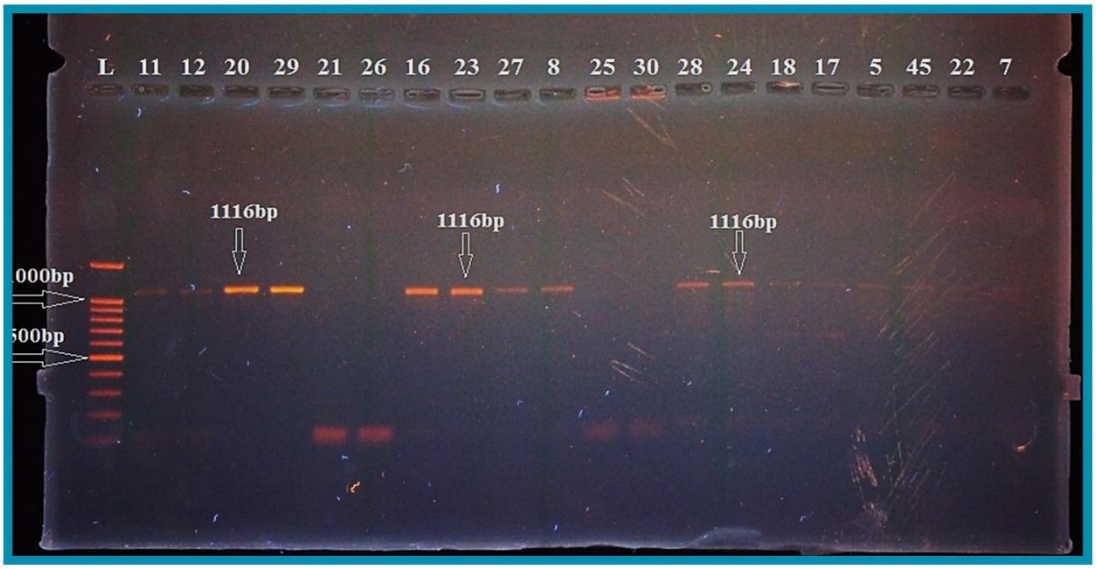

Figure (4) :- Gel electrophoresis for PCR product of (MCR-4 primer) showed 1116bp (Agarose 1%, 10min. at 100 voltage and then lowered to 70 volts, 60min.).Visualized under U.V light after staining with ethidium bromide. Lane L : DNA ladder (100-1500bp), Lanes (11-29 , 16-8 , 28-45 and 7) represented positive results of bacterial DNA isolates, Lanes(21,26,25,30 and 22) represented Negative results lane N represent negative control .

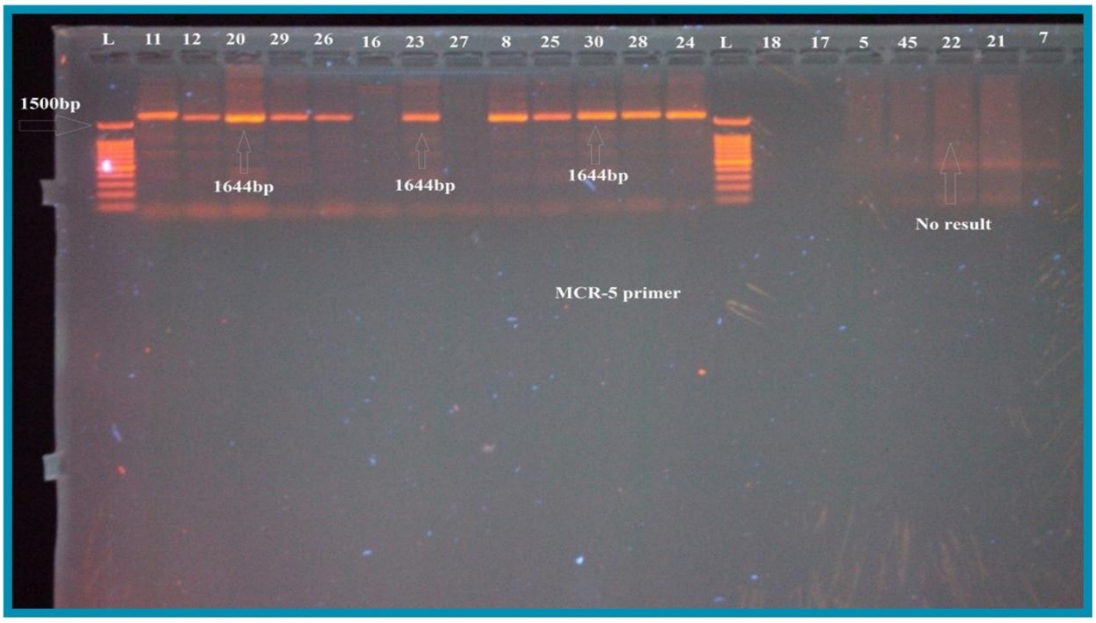

Figure (5) :- Gel electrophoresis for PCR product of (MCR-5 primer) showed 1644bp (Agarose 1%, 10min. at 100 voltage and then lowered to 70 volts, 60min.).Visualized under U.V light after staining with ethidium bromide. Lane L : DNA ladder (100-1500bp), Lanes (11-26 , 23 and 8-24) represented positive results of bacterial DNA isolates, Lanes(16,27 and 18-7) represented Negative results lane N represent negative control .

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Received 16 February 2021; Accepted 08 March 2021.

CONCLUSION

*Acinetobacter baumannii*is important cause of nosocomial infection, showed highlevels of resistance to most antibiotics, therefore, spreading of multidrug resistantbacteria represent a major problem in the area of infection disease. *A.baumannii* were found to be carrycolistin resistance genes in high percentages. The alarming situation with dissemination of resistance genes producing*A.baumannii*isolates, highlights the need for strict antibiotic policy and should be adopted inhospitals to estimate the impactof higher resistance in bacteria and to take steps for reducing thisresistance.

ETHICAL APPROVAL

Verbal consent is taken from each patients before sampling. This study was approved by the Committee of publication ethics at College of Medicine, University of Babylon, Iraq .

ACKNOWLEDGMENTS

All authors would like to express gratitude to all patients who donate by samples that the search was completed.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

None.

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