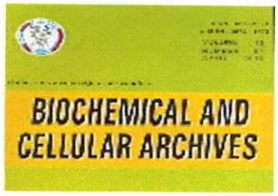

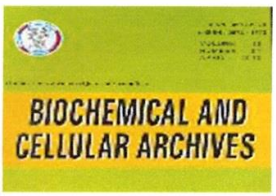

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Acceptance Letter

20 August 2018

Dear Author(s)

Ref.No. BCA Alk 182

Dr./Mr(s). Safaa M. Salman / AL-Qadisiyah University, Iraq

Dr./Mr(s). Azhaar N. Hussien / AL-Qadisiyah University, Iraq

Greetings,

With reference to your article titled:

Identification of Enterobacter cloacae by Using HSP60 Gene and Studying the Minimal Inhibition Concentration and Plasmids Curing

We wish to bring to your kind notice the following

√ **We acknowledge the receipt of the above mentioned article**

√ **The above mentioned article(s) has been sent to the reviewer of expert comments**

√ **The above mentioned article(s) numbered (*BCA Alk 182*) have been accepted and it will be published in the Journal (Biochemical and Cellular Archives ISSN: 0972-5075)**

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Identification of *Enterobacter cloacae* by Using *HSP60* Gene and Studying the Minimal Inhibition Concentration and Plasmids Curing in it.

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Keywords: *Enterobacter cloacae*,*HSP60* gene , MIC , SDS ,Plasmids Curing .

Summary :

A total of 264 specimens from different clinical sources from inpatients of AL-Diwanyah's hospitals were collected. Twenty six isolates were identified as *Enterobacter cloacae* by using *HSP60* gene besides morphological characterization , biochemical tests and use of Vitek System . The minimum inhibitory concentration (MIC) was calculated for the current study isolates and the results showed complete resistance to Amoxicillin and Cephalothin with concentrations ≥ 32 and 32 - 128 mg / ml respectively , While MIC for Cefotaxim was 4 to 32 mg / mL and for Ceftriaxone 4-16 mg / ml. For quinolones antibiotics, all isolates were sensitive to Ciprofloxacin at MIC ≥ 4 mg / ml while 17 isolates showed sensitivity for Nalidixic acid at MIC 32-128 mg/ml .For tetracyclines, the isolates showed absolute resistance to Doxcyclin at MIC 16 to 64 mg / ml and 16 to 32 mg / mL for Tetracycline. MIC ranged from 1 to 8 mg / ml for Chloramphenicol , but for Vancomycin all isolates showed ability to growth at concentrations of 16 - 64 mg / ml . Sodium dodecyl sulfate salts (SDS) were used to investigate about the sites of the antibiotics resistance genes by using curing plasmids method). The plasmid bundles of the treated isolates were

eliminated and the resistance to most of the antibiotics which they resisted it before the treated with SDS ,was lost .

Introduction :

Enterobacter cloacae are genus of Enterobacteriaceae , gram negative, rod shape with small capsule ,its dimensions 0.3-0.6 µm width and 0.8-2.0 µm length and live as a normal flora in human digestive tract[1,2].*HSP60* gene can use as molecular identification tool for *Enterobacter* genus [3]. Most of infections which caused by *E. cloacae* are endogenous as a result of immunity decreasing and opportunistic of these bacteria[2]. *E. cloacae* can cause urinary tract infections , septicemia , bacteremia and enterocolitis[4] . Pathogenic of *E. cloacae* depends on its ability to form biofilms especially whenever using urine and intravenous catheter system and their ability to resist number of antibiotic as penicillin and first three generations of cephalosporin[5,6]. Finding of minimal concentration of cephalosporin in periplasmic space of *E. cloacae* , permeability decreasing of outer membrane and production of β-lactamases enzymes are reasons for its resistance of beta lactam drugs[7]. Some of resistance antibiotics genes are carrying on plasmids which can transfer to the recipient bacterial cell and make it resistant too[8].plasmids can be eliminated by using curing agents as physical agents (heat, radiation) or chemical agents (sodium dodecyl sulfate , ethidium bromide, acridine orange) consequently form bacterial cells missed plasmids which coded for resistance of antibiotic[9].

Materials and Methods

Isolation and Identification :

A total of 264 specimens from different clinical sources from inpatients of AL-Diwaniyah's hospitals in Iraq were collected. The isolates were identified as *E. cloacae* by using morphological characterization of colonies on routine media (Macconkey agar and blood agar) (Difco, USA) and biochemical tests besides using of Vitek 2 Compact System (Biomerieux , USA) as accuracy tool for final identification.

Minimal Inhibitory Concentration (MIC):

Stocks and Ridgway[10]method was followed to calculate Minimal Inhibitory Concentration of *E. cloacae* .Stock solution for each antibiotic was prepared by dissolving 1g of antibiotic in small amount of suitable solvent and the volume complete to 10 ml ,Muller Hinton agar was dispensed into screw cups (20 ml of media for each screw cups)and autoclaved , the media were poured in marked petri dishes .Serial doubling concentrations of antibiotics from 4-1024 µg/ml were prepared by adding known volumes of stock solutions to petri dishes which were contained M.H. media and left at room temperature to become solid. Decimal dilution (10^{-2}) of grown cultures of *E. cloacae* were prepared by using normal saline solution , then 5 µl were pulled up from it with micropipette and were added into prepared petri dishes which were contained of media and antibiotic and incubated at 37°c for 24 h , then results were read and compared with CLSI[11] to determinate MIC of these bacteria.

Plasmids curing :

Sonstein and Baldwin[12] method was used to cure *E. cloacae* plasmids by putting the isolates in test tubes contain Brain Heart Broth media (BHB)(oxoid, USA) and transferred to shaker incubator at 37°c for 24 h .6 grams of Sodium Dodecyl Sulphate (SDS)(B.D.H.,England) were dissolved in 60 ml of distilled water by using screw cups. BHB media were prepared and dispensed in test tube (10 ml for each test tube) , then ascending concentrations of SDS (0.5,1,2,3,4,5,6,7,8,9,10) were prepared. 100µl of bacterial culture added to each tube of SDS and incubated at 37°c for 24 h then got out to observe the tubes in which growth disappeared. Serial of dilutions of bacterial culture were prepared by using normal saline solution (7 tubes were used and each one contain 9 ml of normal saline solution) and 1 ml of bacterial culture of *E. cloacae* was added to the first tube and 1ml from $2^{nd}$to 3^{rd}till the 7^{th} tube. 100µl of each dilution were spread on MacConkey Agar dishes and incubated at 37°c for 24 h. 100 pure and isolated colonies were selected and transferred with sterile loop to dishes of Muller Hinton agar dishes contain antibiotic which were bacteria resisted it before the curing plasmids (if there is a growth after incubation period on these dishes that means losing of resistant character of treatment bacteria).

DNA Plasmid Extraction : High-Speed Plasmid Mini Kit (Geneaid, South Korea)was used to extract DNA plasmid depending on procedures which were found in this kit . Extraction produces were observed by using electrophoresis on agarose gel and U.V. ray .

Amplifying of *HSP60* gene:

Polymerase Chain Reaction (PCR) was used to amplified heat shock protein *(HSP60)* gene .PCR mixture which contains from : Prime Taq Premix (GeNet Bio , South Korea) , primers of *HSP60* (table 1), DNA template and free nuclease water and the Cycle conditions were: 30 cycles , denaturation at 94˚C for 30 sec., annealing at 57.5˚C for 30 sec. and extension at 72˚C for 60 sec. and produces of amplifying for *HSP60* gene were showed by using agarose gel electrophoresis and U.V. ray.

Table 1. primers which were used in this study

gene	PCR primer	Primer sequence 5' _3'	Tm °C	PCR product (nt)	Reference
HSP60	Hsp60-F	GGT AGA AGA AGG CGT GGT TGC	61.8	341	8
	Hsp60-F	ATG CAT TCG GTG GTG ATC ATC AG	60.6		

RESULTS and DISCUSSION :

A total of 26 isolates of *E. cloacae* were got from 264 specimen from different clinical sources including urine , stool , blood and urine catheter system . Identification was depended on their cultural characters, microscopic , biochemical test (table 2) and using of Vitek 2 Compact system . Amplifying of *HSP60* gene by using PCR method (figure 1) was used to ensure that these bacteria are follow *Enterobacter* sp.

Table 2. Biochemical tests of *E. cloacae*

*test	Indole	Oxidase	MR	Urease	Citrate	VP	H_2S	Gas	Motility
Rate of negative isolates (%)	100	100	100	84.6	7.7	26.93	100	34.62	-
Rate of positive isolates (%)	-	-	-	13.4	92.3	73.07	-	65.38	100

*All isolates were Acid/ Acid for kligler test , MR= methyl red , VP= Voges Proskauer

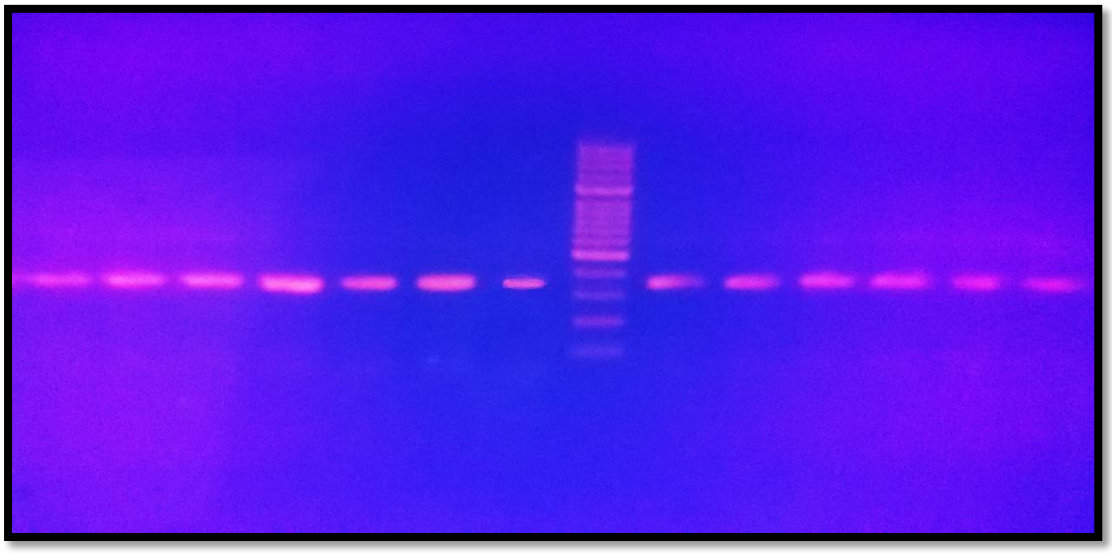


Figure 1. Electrophoresis and produces of amplifying for *HSP60* gene (341 bp) , agarose 1% and using 80 V for 1 hour ,DNA marker 100-3000 bp

From the results we found that the rate of our isolation of *E. cloacae* was 9.84% dispensed as showed in (table3) .The total rate of isolation was similar to [14] who get 9.61% of this bacteria including two subspecies (*E. cloacae* subspecies *cloacae* and *E. cloacae* subspecies *dissolvens*) .The catheter systems are gates to entre *Enterobacter i*nto blood stream[15], and the role of *Enterobacter* as causing of diarrhea is common because these bacteria are opportunistic and can transfer from normal flora to be pathogenic and striking the human body and its tissues especially when make surgical operations or put the urine catheter systems for a long time which can contribute in spreading of bacteria and causing Nosocomial infections[16].

Table3: Sources of specimens and the rate of isolate for each one

Type of specimen	The case	Number and percent of specimen (%)	Number and percent of isolates(%)
Urine	UTI	77 (29.16)	14 (5.3)
stool	Diarrhea	90 (34.09)	5 (1.8)
***U.C.S**	Surgical operations	58 (21.96)	3 (1.13)
****U.C.T swabs**	=	38 (14.39)	3 (1.13)
Blood	Bacteremia	1 (0.37)	1 (0.37)
Total		264 (100)	26 (9.84)

*** U.C.S=urine catheter sacs , **U.C.T= urine catheter tubes**

MIC Calculating :

The results of MIC Calculating of our isolates (table4) showed that all our isolates were resisted amoxicillin at concentrations 32-256 µg/ml and this result is similar to [17]who was found that all bacteria followed enterobacteraceae including *Enterobacter* sp. are resistant to amoxicillin because of its ability to produce chromosomal β-lactamases enzymes type AmpC and the next generations can acquire this resistance .MIC of cephalosporin antibiotics showed full resistance to cephalothin at concentrations 32-128 µg/ml , 15 isolates (57.69%) were resisted Cefotaxime at concentrations 4-32 µg/ml while 9 isolates were resisted Ceftriaxone at concentrations 4-16 µg/ml. The ability of *Enterobacter* to resist cephalosporin antibiotics including 3rdgeneratin due to wide using of these antibiotics for treatments or happening of mutations caused excessive production of AmpC β-lactamases besides of acquirement new genes coded for β-lactamases by horizontal transferring (plasmids , transposons and bacteriophages)[18,19]. Results of MIC of Quinolones as Ciprofloxacin and Nalidixic acid which were used in this study was at concentrations ≤ 1 µg/ml for ciprofloxacin (all isolates were sensitive to it) and 32-128 µg/ml for Nalidixic acid (17 isolates were sensitive to it) . Quinolones are antibiotics describe widely for treatment many of bacterial infections because of its bactericidal effect and safely using but recently , bacterial communities showed resistant against these antibiotics due to change of amino acids as result of mutation which happened in positions responsible for quinolones resistant besides *E. cloacae* contain two types of plasmids (QnrS and QnrA)which are responsible for protection of bacterial DNA and blocking it from binding with Quinolones[20]. All isolates showed full resistance to doxcyclin at concentrations 16-64 µg/ml while 19 isolates (73.07%) were resisted tetracycline at concentrations 16-32 µg/ml and the rest isolates were intermediate to it. This result agrees with[21]in their study on types of *Enterobacter* and its resistant to

doxcyclin but it disagrees with them about MIC of tetracyclin and this difference may be due to differences among the types of these bacteria . The resistant of tetracycline group depends on efflux pump or decreasing of outer membrane proteins which are associate with permeability of these antibiotics into bacterial cell [22,23] . Our isolates showed absolute resistance to Chloramphenicol at concentrations 1-8 µg/ml and this result disagrees with [24]who found some isolates resistant to chloramphenicol and this difference is due to difference of isolation source where part of their isolates from clinical sources and another from environmental sources , or due to differences among the types of *Enterobacter*. High sensitivity of chloramphenicol maybe due to their extended spectrum (bacteriostatic) which inhibit bacterial protein synthesis as a result of binding with subunit 50S and preventing peptidyl transferase enzymes from adding new amino acid which are necessary for elongation of polypeptide chain[24]. All isolates were resistant to Vancomycin at concentrations 16-64 µg/ml and the result agrees with [21, 25]who were found in a study of Sepsis outbreak in neonatal Intensive Care Unit that vancomycin was inactive toward *E. cloacae.* Part of resistant to vancomycin result from finding *vanA and vanB* in bacteria which follow enterobacteraceae family and these genes cause resistant of glycopeptide group which include vancomycin antibiotic and these genes code for alternative pathway of cell wall biosynthesis and decease the ability of contents for binding with vancomycin[26].

Table 4 : MIC and the number of resistant isolates

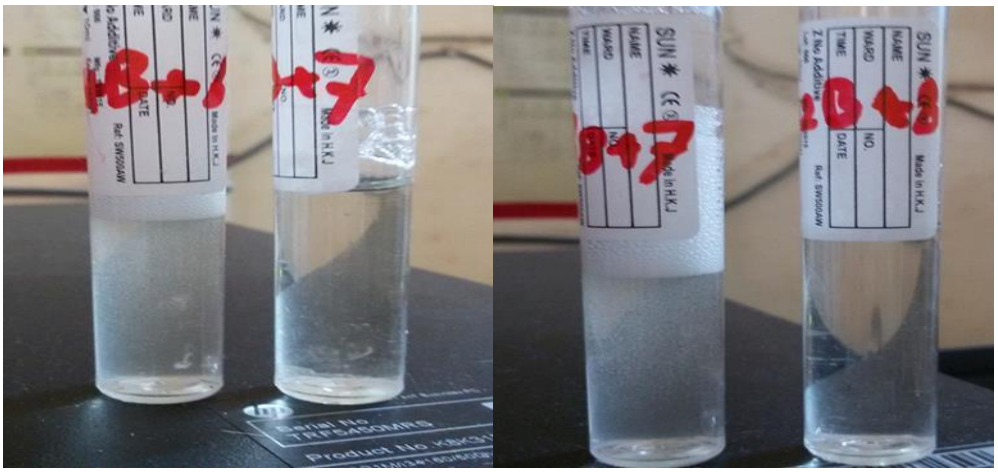
antibiotic	MIC according to CLSI 2017			MIC (mg/ml) for our isolates and the number of resistant isolates								
	S	I	R	4	8	16	32	64	128	256	512	1024
AMO	≤ 8	16	≥32				26	21	12	7		
CRO	≤ 1	2	≥4	9	7	2						
CTX	≤ 1	2	≥4	15	11	4	1					
CEP	≤ 16	-	≥32				26	19	8			
TET	≤ 4	8	≥16			19	6					
Dox	≤ 4	8	≥16			26	12	4				
CIP	≤ 1	2	≥4	-	-	-	-	-	-	-		
NA	≤ 16	-	≥32				9	4	1			
C	≤ 8	16	≥32	-	-	-	-	-	-	-		

Van	≤ 2	8-4	≥16			26	17	9				

AMO=Amoxcillin , CRO= Ceftriaxone , CTX= Cefotaxime , CEP=Cephalothin, TET= Tetracycline , Dox= Doxcyclin , CIP= Ciprofloxacin , NA= Nalidixic acid , C= Chloramphenicol , Van= Vancomycin .

Plasmids curing :

Plasmids curing assay were executed on two isolates (9 and 21 isolates) which were selected randomly among 26 isolates were collected in this study to know the role of plasmids in resistance of antibiotics (R-plasmids)by removing (curing) the plasmids which code to antibiotics resistant .This assay depends on idea that failing of bacteria to grow in media contains antibiotics which grown on it before plasmids curing , means the character of antibiotics resistant carried on plasmids but if bacteria grew after curing plasmids that mean the character of antibiotics resistant carried on another genetic elements .For curing assay we can use number of curing agents which can be either physical factors (high temperature . U.V) or chemical factors (orange acridine , ethidium bromide , Sodium Dodecyl Sulfate (SDS)). IN this study we used SDS material as a curing agents in doubling ascended concentrations from 0.5-10% .Results showed that highest concentration of SDS which isolates (9 and 21)could grow in it , was 7% and 6% respectively (figure 2),and 100µl from each these test tubes spread on MacConkey agar dishes with sterile swab and incubated at 37°c for 24 h. Pure and good isolating colonies selected and inoculated on Muller Hinton agar dishes contain antibiotics which these isolates resisted it before plasmids curing and incubated again at 37°c for 20-24 h. Dishes put out and we observed failing of these two isolates to grow again on the same dishes which grew on it previously,(table 5).



A **B**

Figure 2 : A- growth of isolate No.21 in 6% SDS , B- growth of isolate No.9 in 7% SDS

Table 5 : susceptibility Isolates 9 and 21 for Antibiotics before and after plasmids curing

No.	Antibiotic	Isolate No.9		Isolate No.21	
		Before	After	Before	After
1	Ceftriaxone	+	–	+	–
2	Cefotaxime	+	–	+	–
3	Cephalothin	+	–	+	+
4	Nalidixic acid	+	+	+	–
5	Tetracycline	+	–	+	–
6	Doxcyclin	+	–	+	–
7	Amoxicillin	+	+	+	+
8	Vancomycin	+	–	+	–

Resistance = + , susceptible = –

DNA Plasmid Extraction was executed for 9 and 21 isolates before and after curing process (figure 3),on agarose gel and we observed of one plasmid band for each isolate before curing and it disappeared after curing process. The disappearing plasmid bands explain reasons of resistant's losing of some used antibiotics from treated isolates while the resistant of another antibiotics as amoxicillin remained after curing and this mean that resistant genes carried on another genetic elements as chromosome. Plasmids can be lost during bacterial cell division where [27]showed that plasmids trans genetically from parents to new individuals during cell division but not dispensing among filial equally or losing sometimes .

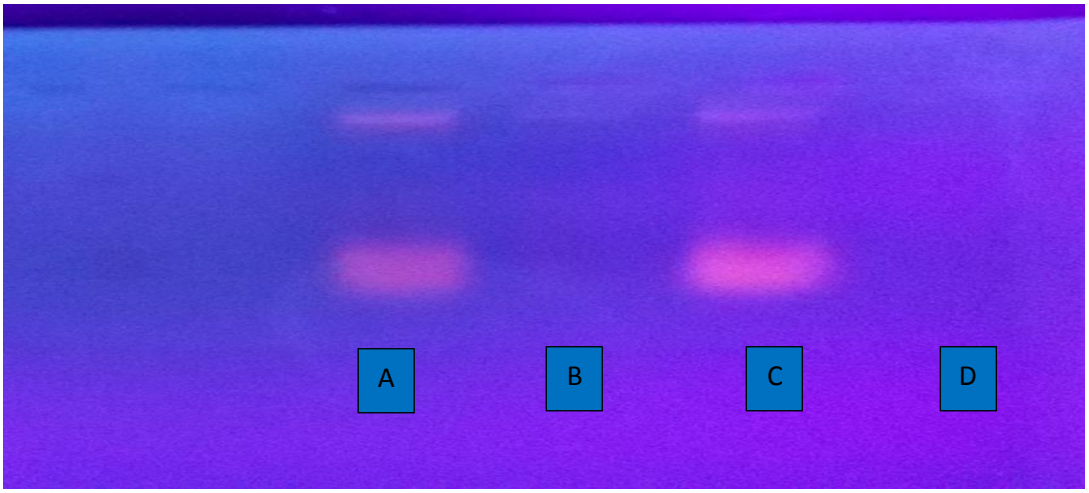


Figure 3 : Electrophoresis of DNA Plasmid of 9 and 21 isolates of *E. cloacae* by using 1% agarose , 80 v for 1 h . (A , C) showed plasmid content of 9 and 21 isolates before curing process while (B,D) after it.

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