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Molecular study of some virulence factors encoding genes of Enterobacter spp. Isolated from different clinical specimens

Mahdi.Hussain .Muheel Alammar

Kufa University, Faculty of Sciences-Department of Biology, Iraq[2]

Corresponding Author**: Adress:** Kufa University, Faculty of sciences-Department of Biology, Najaf, Iraq phone:07802424739 *Email*- dr.mahdi65@ymail.com

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Abstract

The study aimed to isolation and identification of *Enterobacter* spp. from different clinical infections ,as well as virulence factors encoding gene were detected by PCR.The clinical specimens (100) were collected from patients suffering from different infectious from various hospitals of Najaf during the period from February 2011 to June 2012. *Enterobacter* isolates which were diagnosed according cultural and biochemical tests as well as API 20E .

Eighty four isolates of *Enterobacter* were collected, from different samples which distributed in two species of *Enterobacter* , *E.cloacae* (89.3%) and *E.sakazakii* (10.7 %) . The results showed that all isolates were produced of siderophore , and most isolates were capsule formation , but give negative result for haemolysin and protease . Also, 80 (95%) of *Enterobacter* has the CFA / III and 15(18 %) of isolates are bacteriocin production.

The results showed that 26 (31%) of isolates were had *rpo*S gene , and these isolates were more resistance to stress conditions which lack the gene . The resistance was high in stationary phase than that in log phase. The result showed that all isolates were contained nitroreductase gene, but not had shiga like toxin gene.

Introduction:

Enterobacter spp. belonging to the family Enterobacteriaceae which are facultative anaerobic gram – negative bacilli, most species are motile with flagella and have class 1 fimbriae.They produce acid upon glucose fermentation , methyl red negative , and Voges-Proskauer positive , with an optimal growth temperature 30˚ C, 80% are capsulated [1].

In human, multiple *Enterobcter* species are known to act as opportunistic pathogens, including *E. cloacae*, *E. aerogenes* , *E .sakazakii* ,*E . gergoviae* , and *E . agglomerans* . *Enterobacter* spp. which can cause numerous infections , including eye and skin infections , meningitis , bacteremia , pneumonia , urinary tract infections , wound , intestinal infections and surgical site infections[2] .

Enterobacter involved in extra intestinal infections, which are known to possess virulence associated characteristics, they have the ability to adhere to and invade eukaryotic cells, once outside the gastrointestinal tract, they would take advantage of being able to chelate iron to survive and spread within the host .In enteric bacteria one of seven additional factors (Sigma –factors) bind RNAP (the complex is termed RNAP holoenzyme) and confer promoter recognition of specific groups of genes (termed regulons

The sigma factors were σ70 RpoD(growth related/housekeeping), σ54 RpoN

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(nitrogen utilization), σ38 RpoS(Stress general), σ32 RpoH(Heat shock response), σ28 RpoF(flagellar synthesis; chemotaxis))[3] .

When RPoD is abundant in diving cells , during this time , RpoS is barely detectable ,but protein abundance dramatically increases in response to unfavorable growth conditions). RpoS is also involved in the process of virulence of some pathogens . Intact, some of the regulated genes that are expressed in response to starvation are also considered virulence factors, such as HPII catalase, which confers resistance to H_2O_2[4] .

The nitroreductases proteins form a group of enzymes that have a central role in the reduction of nitro groups on nitro compounds .Type I (oxygen-insensitive) nitroreductases catalyze the sequential transfer of two electrons from NAD (P) H to the nitro groups (Spain, 1995) and Type II (oxygen-sensitive) nitroreductases catalyze one-electron reductions of the nitro group in the presence of oxygen[5].

SLTs are capable of causing diseases including diarrhea , hemorrhagic colitis and hemolytic – uremic syndrome in humans. Much of the focus of identification and characterization of shiga toxin has been on *E. coli* O157 :H7 strains , [6.The toxin has also been observed in other bacterial genera, including *Citrobacter* , *Enterobacter* .In 1996 reported the isolation of a strain of *Enterobacter cloacae* producing SLT-2 from the feces of patient with hemolytic – uremic syndrome [7].

The incidence of infection caused by *Enterobacter* spp. is increasing and littleness studies related to its ability to produce the virulence – associated properties , for this reason, the aim of study was achieved by detected some virulence factors genes such as RpoS (σ factor) , nitroreductase gene and shiga like toxin (SLT) gene by using molecular methods (PCR).

- Methodology:

1: Samples collection :A total 100 different specimens were collected from patients who attended to various hospital of Najaf city (IRAQ) with different clinical infectious , during the period from February 2011 to June 2012. A single colonies were isolated from primary positive cultures and identified according to the criteria of [8,9,10,11] .

2. Identification of Entrobacter sp.

- Cultural Characteristics : Gram's stain was used to examine the isolated bacteria for studying the microscopic properties as initial identification of Entrobacter sp. [10]. Morphological colonies characteristics were recorded on the specific media for primary identification of Entrobacter sp.. 1-2 colony of Entrobacter sp. were tested for oxidase, catalase, Simmone Citrate, Kligler Iron agar and Indole tests and all these tests positive [11].In addition to Entrobacter sp. identification By using API 20 E (Biomerieux, France).Entrobacter sp genes. virulence detection of Phenotypic and genotypic-

Phenotypic detection of Entrobacter sp were done according to[8,9] and assembling PCR materials were made according to the manufacturer's instructions (Promega, Madison, USA) using PCR reaction mixtures prepared in a 0.2 ml Eppendorf tube with 25 full reaction volumes, which contained the following: 12.5 III Go Taq® Green Master Mix X2 (Promega, Madison, USA)., 2.5 III upstream primer, 2.5 III downstream primer (synthesized by Alpha DNA Company, CITY, Canada), 5 III DNA template, 2.5 III nuclease-free water . All the appending was handled in a laminar flow on ice. Entrobacter sp DNA templates were examined by PCR using eight sets (F and R) of primers targeting genes to determine the virulence factors. PCR for detecting the gene representing primers were ,*nt* F [5- GCGCTCATCACCAGGAGTTGCCAT GG ATA-

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3],R[5-GCGCGCCAGCGTGCCAGAGGAGCTCA-3'-],*rpoS*.F[5-CTGAA TTC CC TGAGTTGCCTACGCCC-3] R[5-CTTAATCAG GAAGGGGGGGAATT CGTG 3], *slt* F[5-ATACAGAG(GA)G(GA)ATT TCG T-3] R [5-TGATG ATG (AG) CAA TTCAGTAT-3 [12.13].

The reaction mixture was prepared by mixing the components in sterile Eppendorf tubes (0.5 ml) , which were then centrifuged at 30 minutes, then the Eppendorf tubes were closed and transferred into a Thermo cycler. Buchi (Switzerland)For product detection, 5 µl of the PCR mixture was subjected to electrophoresis in a 2% agarose gel The reaction mixture contained Go Taq® Green Master Mix, X2, which is a premixed ready-to-use solution containing bacteriologically derived Taq DNA polymerase dNTP, MgCh, and reaction buffers at optimal concentrations that is recommended for any amplification reaction to be visualized by agarose gel electrophoreses and ethidium bromide staining [12].

A-Genotypic detection of RpoS

Stationary –phase cultures were prepared by overnight growth of bacterial in LBB medium (5 ml of volume) and incubated overnight at 37 ˚ C ([13] and Genomic DNA was extracted according to Geneaid company instructions , DNA concentration was measured according to the method [14].

B.Detection of nitroreductase gene

All isolates of bacteria that were positive to nitrate reduction were tested to detection of nitroreductase gene by PCR as follows:

Bacteria were suspended in 5ml of LBB media and incubated overnight at 37˚ C. This culture was used to inoculate 1L of LBB, which was growth at 37˚ C until the optical density at 600 nm had reached 1.0 , the bacteria were collected by centrifugation [12 and genomic DNA was extracted according to Geneaid company instructions , DNA concentration was measured according to the method Sambrook & Russel (2001) [14].

C. Detection of shiga like toxin gene

It was used modified method of Maniatis *et al*.[15][for examination of fecal cultures by PCR technique , approximately 500mg of feces was inoculated MacConkey agar and incubated at 37˚ C for overnight . If bacteria that grown on this media was identified as *Enterobacter* spp. .It was transport this bacterium from MacConkey agar to 10 ml of LBB medium and incubated at 37˚ C for overnight, then, sample was prepared for DNA extraction

4-The relation of RpoS with environmental conditions

All isolates that were possessed and not possessed rpoS gene were tested of their stress resistance as followed:

Stationary –phase cultures were prepared by overnight growth of bacterial in LBB medium at 37 ˚ C. Exponential –phase cultures were prepared by diluting 1: 1,000 overnight cultures in LBB fresh medium and incubating them for several hours at 37˚ C until a Do600 0.3-0.5 was achieved.

Stationary – phase survival was analyzed in LBB at 37˚C with vegrous shaking, resistance to different stress conditions (heat and osmolarity) was checked in exponential and overnight cultures . Cells diluted to final cell density of 5,000 -10,000 cfu ml -1with physiological saline solution were exposed to heat (51˚ C) at 5, 10 and 15 minutes and incubated overnight at 37˚ C , 5 M NaCl at 2,4,6 and 8 hours and incubated at 37˚ C, the number of surviving cells at different times determined , o.1ml aliquots were plated on LB agar . 100% viability was considered to the number of bacteria at t =0 [13] .

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The results and discussion

- *Enterobacter* isolation and Identification

The results indicated of 84 isolates of *Enterobacter* spp. were collected from various samples. All *Enterobacter* spp. (*E.cloacae* and *E .sakazakii*) were appeared slightly pink colonies (weak lactose fermentation) ,that were round , 2-3 mm in diameter and dark center ,slightly mucoid less than *Klebsiella* , with regular edges, while *E.sakazakii* was formed irregularly round colonies , rough and wrinkled colonies, which are difficult to remove with a platinum wire.Two species of *Enterobacter* were grew efficiently on CHROM^{TM} agar medium and yielded metallic blue colonies Fig. 1 [16] .

E.cloacae

CHROM agar

E. sakazakii

Figure(1) : *Enterobacter* colonies on CHROM aga media

The results showed, that (*E.cloacae* and *E .sakazakii*) were gave positive result to simmons citrate , Voges Proskauer, catalase tests and negative to methyle red , H_2S production and oxidase test , all isolates of *E.cloacae* were negative to indole test , while *E. sakazakii* were positive ([9,17].

It was founded only three isolates of *E .sakazakii* could produce gelatinase , and one isolates was fermented D-sorbitol ,in contrast all isolates of *E.cloacae* were fermented this sugar . In related with malonate utilization , 88% and 22% of *E.cloacae* and *E.sakazakii* were utilization malonate respectively [1] .

- Virulence factors of *Enterobacter* species

The results of virulence factors of *Enterobacter* isolates,table -6 show that all *Enterobacter* didn't produced any type of hemolysin and extracellular protease, [18] but all isolates of *Enterobacter* species had a polysaccharide capsule surrounding the bacterial cell except one isolate of *E.cloacae* that lack capsule structure (98.8% versus 1.2%) [19] .On the other hand, all isolates of *Enterobacter* have the ability to produce siderophore 84 (100%) [20] have suggested that aerobactin influences either the extent of bacterial translocation from the intestinal tract, the extent of bacterial multiplication in tissues following translocation, or both. Thus, aerobactin secretion *in vivo* could be an important step in the stages of the infection cycle during which intestine – population opportunistic bacteria effectively colonize the gut, penetrate the mucous layer covering the intestinal villi , translocate out of intestinal lumen through the epithelial cells , and finally spread to organs within which they may survive. In the results they appeared all isolates of *E.sakazakii* are able to produce siderophore , this

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virulence factor may be associated with ability of bacteria to causes necrotizing enterolcolitis

A 32 % of isolates were had ability to produce CFA / I , and 10 % of isolates able to produce CFA /II, which causes agglutination of chicken blood, and act to adhere of bacteria with specific and complex carbohydrate receptors of epithelial cells of small intestine [21] , whereas *E. Sakazakii* isolates were negative result .But 95% of *Enterobacter* isolates to produce of CFA / III,. The search for the production of colonization factor antigens among *Enterobacter* isolates in this study has demonstrated that more isolates were possessed CFA/ III compatible with the presence of type 3 fimbriae (mannose – resistant fimbriae) .This factor is responsible for colonization the surfaces of indwelling devices (e.g. urinary catheters) [22] .

The relationship between CFA / III and pathogenisity of bacteria was established from adherence of bacteria in mucous surfaces or epithelial cells of genital , urinary , respiratory and gastric tract [23].

Table(1): Virulence factors of *Enterobacter* species

Virulence factors		***E.cloacae* n= 75**		***E.sakazakii* n=9**		**No. & (%) of all +ve isolates**	**No. & (%) of all -ve isolates**
		No.& (%)of+ve ve isolates	**No.&(%) of -ve isolates**	**No.&(%) of+ve isolates**	**No.&(% of-ve isolates**		
hemolysin		0 (0%)	75 (100%)	0 (0%)	(100%)	0 (0%)	4(100%)
Capsule		4(98.7%)	1(1.3%)	9(100%)	0 (0%)	83(98.8%)	1(1.2%)
Extracellular Protease		0 (0%)	5(100%)	0 (0%)	(100%)	0 (0%)	4(100%)
Siderophore		5(100%)	0 (0%)	9(100%)	0(0%)	84(100%)	0 (0%)
CFA	CFA / I	21 (28%)	54(72)	6 (66.7%)	3(33.3%)	27(32%)	57(68%)
	CFA / II	8 (11%)	67(89%)	0(0%)	9(100%)	8 (10%)	76(90%)
	CFA/III	71(95%)	4(5%)	9(100%)	0(0%)	80(95%)	4(5%)
Bacteriocin	*	9(12%)	66(88%)	6(66.7%)	3(33.3%)	15(18%)	69(82%)
	**	0(0%)	75(100%)	0 (0%)	9(100%)	0 (0%)	84(100%)

* : *E.coli* , ** : *Klebsiella pneumoniae*

It was founded, that 15 isolates (17.9%) of *Enterobacter* were able to produce bacteriocin , represented by 9(12%) isolates of *E.cloacae* and 6(66.7%) of *E.sakazakii* , while it was found that the bacteriocin *Enterobacter* isolates not affect on *Klebsiella pneumoniae*. Bacteriocin are antimicrobial peptides with different sizes, microbial target and mechanisms of action produced by large variety of bacteria. They are characterized by a bactericidal or bacteriostatic activity against strains of the same species or closely related species and differ from most therapeutic antibiotics due to their narrow activity spectrum and their proteinaceous nature [24].

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It was found low percentages of *Enterobacter* isolates (*E. cloacae* , *E. sakazakii*) are able to produce bacteriocin only against sensitive bacteria *E.coli* . This low production of bacteriocin may be due to siderophore production that can inhibit the activity of bacteriocin such receptor for the uptake siderophore also functions as receptor for the bacteriocin (cloacin)[18].*Enterobacter* bacteriocin were active against *E.coli* , but were inactive against *Klebsiella pneumoniae* , this may be due to the growth inhibitor products of the *Enterobacter* strains for typing are different in nature [18,24].

-Production of RpoS

A-Detection of *rpoS* gene in *Enterobacter* spp. by PCR.

Twenty six isolates (31%) out of 84 isolates of *Enterobacter* were possessed *rpo*S gene, that distributed to 21(28%) isolates of *E.cloacae* and 5 (56%) of *E.sakazakii* table - 2 . RpoS a conserved stress regulator that plays a critical role in survival under stress conditions . It is also involved in virulence of Many pathogens [25]

Table (2 **)** : *rpo*S gene of *Enterobacter* isolates

Enterobacter species	No. isolates	No.& (%) of positive isolates	No.& (%) of negative isolates
E.cloacae	75	21(28%)	54(72%)
E.sakazakii	9	5(56%)	4(44%)
Total	84	26(31%)	58(69%)

A discrete PCR product of the expected size (800 bp) was seen for templates fig- 2. Therefore, the role of sigma factor in the virulence process could be related to its ability to induce genes that allow bacteria to survive under harsh conditions [26]

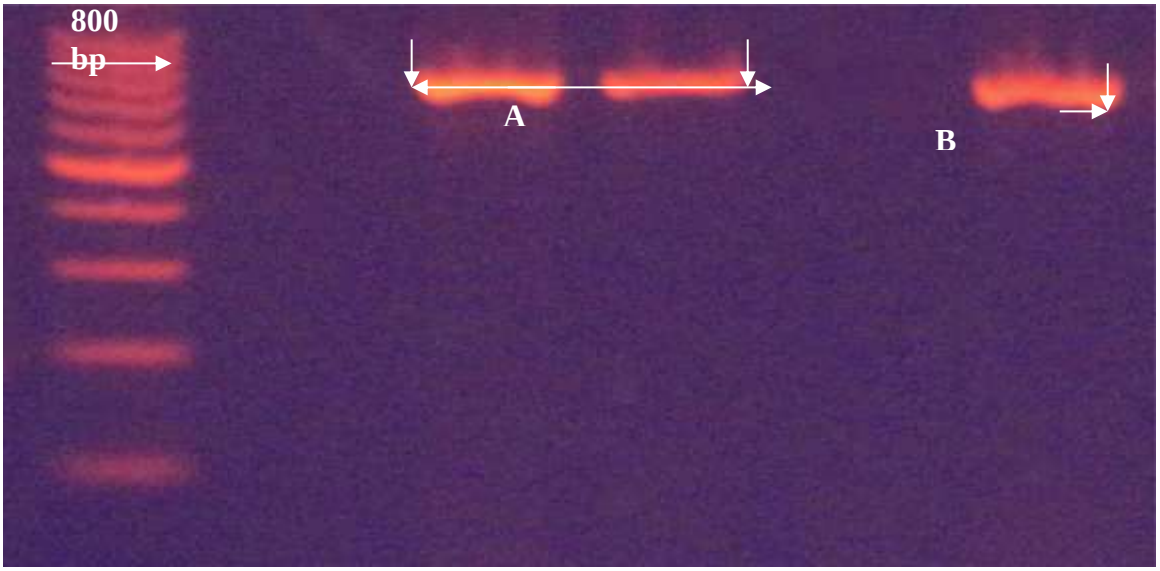

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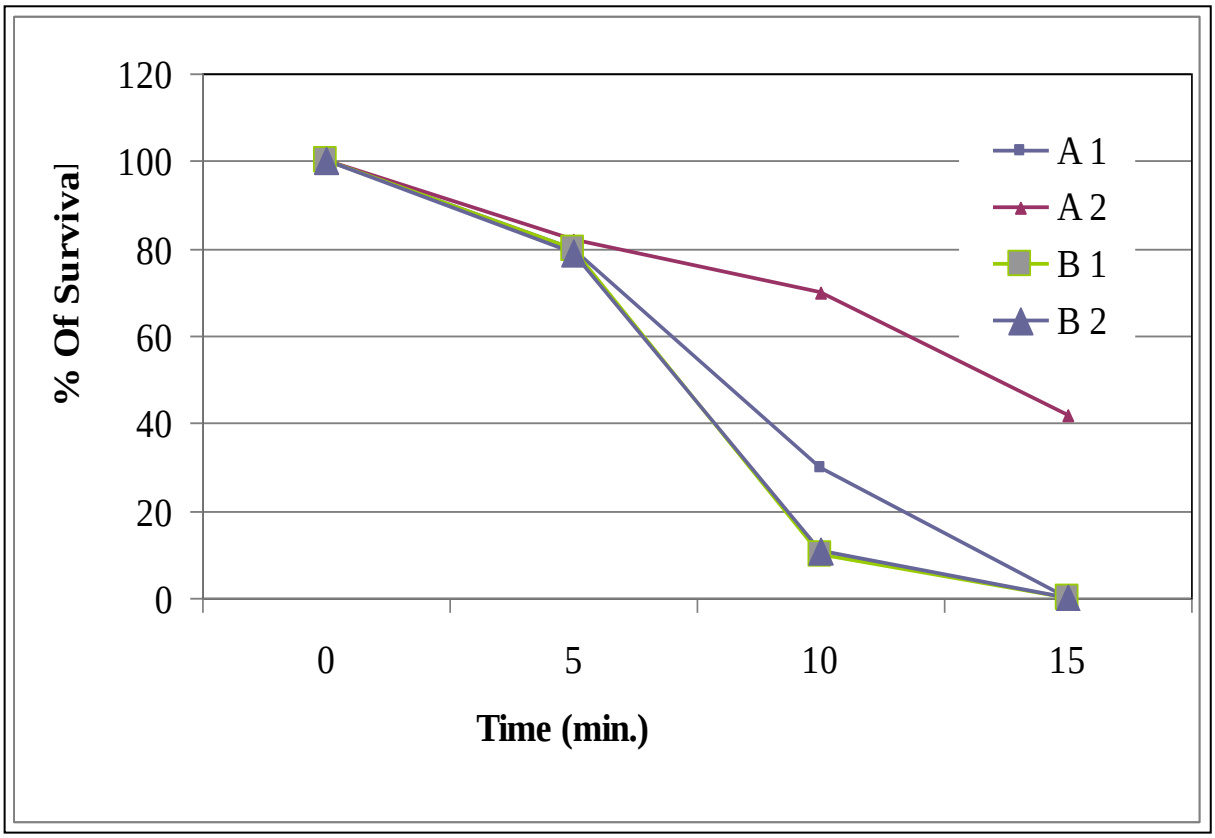
Figure (2) : PCR detection of *rpo*S gene in *Enterobacter* spp. . M: Marker (800 bp DNA ladder) ;A: *E.cloacae* templates , B : *E.sakazakii* template.

B-The relation of RpoS with environmental conditions

It was appeared from figure- 3, the percentages of survival *E.cloacae* that have *rpo*S gene to 51˚C in exponential phase, were decreased with increase the period of exposure, and were reached to 0% after exposure to 15 min. In stationary phase , it was revealed the decreasing of number of survival cells was low than in exponential phase and 2.10 x 10^3 cfu / ml of cells were survived in time 15 min. of exposure, while tolerance much lower in the *rpo*S negative isolates during the stationary phase ,and the statistical analysis was appeared presence of significant differences(≤ 0.05) among *rpo*S positive and negative isolates .

Figure (3): *E.cloacae* stress response to temperature 51◌̊C , A1: Exponential phase of *rpo*S positive isolates , A2 :Stationary phase of *rpo*S positive isolates, B1: Exponential phase of *rpo*S negative isolates ,B2: Stationary phase of *rpo*S negative isolates.



In figure (4) , was appeared, that survival cells of *E.sakazakii* after exposure time 15 min.to 51˚C in exponential phase was 20% and 60% in stationary phase and the numbers of cells were 1 x 10^3 cfu / ml and 3 x 10^3 cfu / ml respectively , the tolerance of *rpo*S negative isolates of this bacteria in two phases were lower than that in *rpo*S positive isolates , and it was appeared the existence significant differences(≤ 0.05) among *rpo*S positive and negative isolates .

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Stationary phase cells of *E.cloacae***.** and *E.sakazakii* exhibited a higher resistance against heat (51˚C) than that exponential –phase cells with *rpo*S positive isolates .and *E.sakazakii* (51˚C) in all times was higher than that in *E.cloacae***.** These results identical with those obtained by[27] who found resistance of *E.cloacae* and *E.sakazakii* to temperatures ranged between (15-55) ˚C , *E.sakazakii* isolates were more grow in high temperature , and he was explained his results to presence enzymes and proteins in plasma membrane were resistant to those temperatures.

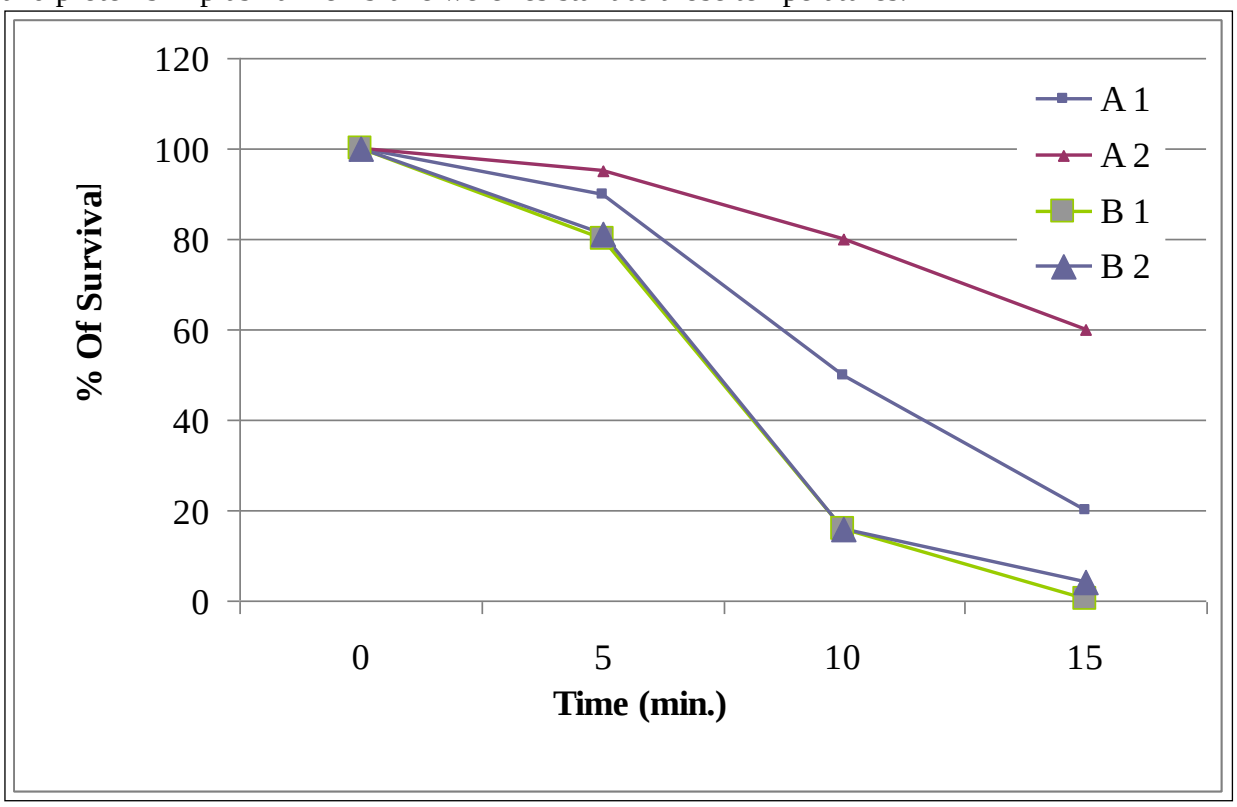

Figure (4): *E.sakazakii* stress response to temperature 51̊C .

After exposure to osmotic stress , it was appeared the percentage of survival cells in stationary phase was higher than that in exponential phase and the numbers of cells were decreased with increased of exposure period, after 8 hours of exposure to 5 M NaCl, the *rpo*S negative isolates were less tolerance to osmotic stress than that in *rpo*S positive isolates with the presence of significant differences($\leq$ 0.05) among the numbers of bacteria (with or without *rpo*S gene figure -5,6 . The numbers of cells that were survived after exposure to 8 hours in 5 M NaCl were 6 x 10^3 cfu / ml and 3.11 x 10^3 cfu / ml in exponential phase and stationary phase in respectively.

The resistance of two species that have *rpo*S gene against osmolarity were high in stationary phase than exponential phase , and appeared , the tolerance of *E.sakazakii* to 5M NaCl in two phases was higher than that in *E.cloacae*. The results were appeared resistance of two species of *Enterobacter* that possess *rpo*S gene resistant to high salt concentration , this resistance is important to survival, colonization and infection processes [28].

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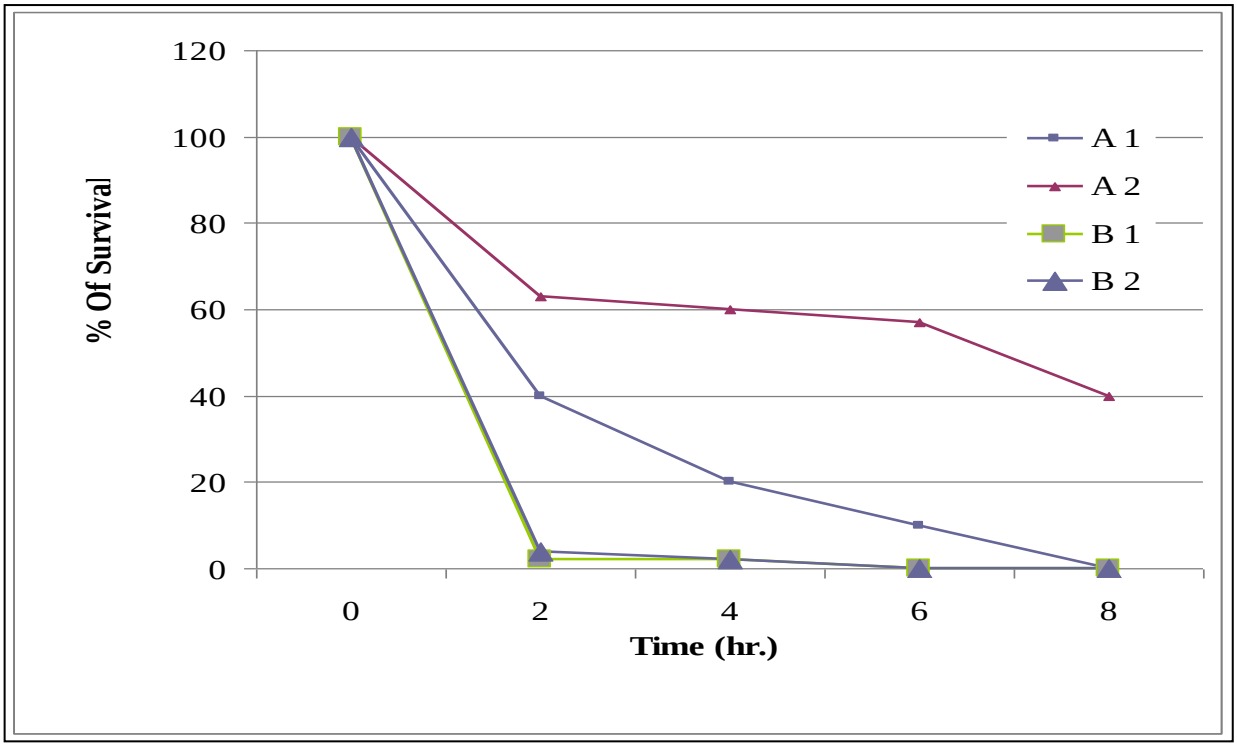

Figure (5): *E.cloacae* stress response to 5M (NaCl) .

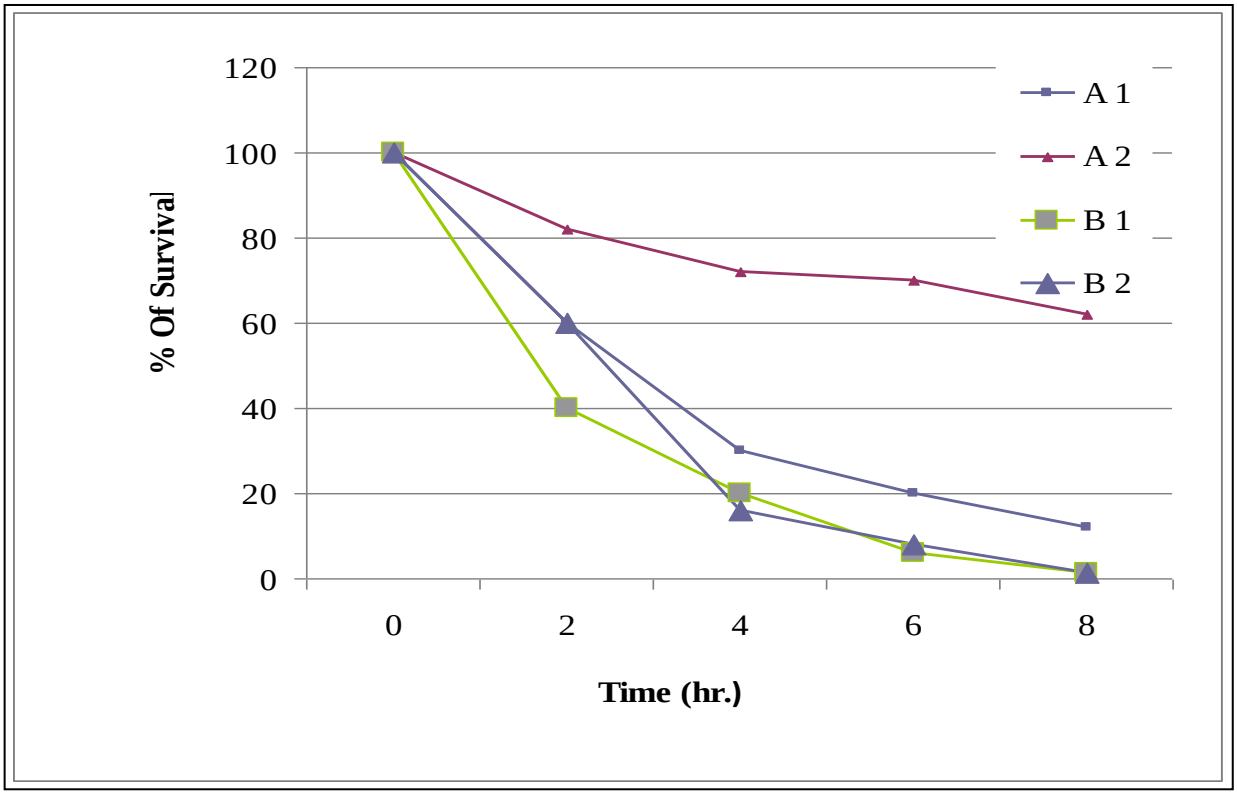

Figure (6): *E.sakazakii* stress response to 5M (NaCl) .

C--Detection of Nitroreductase (Phenotypic & genotypic detection)

The results of nitrate reduction test revealed that , all isolates (100%) of *Enterobacter* were able to reduction nitrate to nitrite**.** The results of PCR detection of nitroreductase gene, indicated that a all isolates of *Enterobacter* (100%) were

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possessed nitroreductase gene. A discrete PCR product of expected size (654 bp) was seen for all templates (Fig 7). Because *Enterobacter* spp. as a part of intestinal microflora , the nitroreductases present in this intestinal bacteria may play a key role in the metabolism of the exogenous nitro aromatic chemicals to which the host is exposed . The products of these biotransformation can be toxic to the host [29] .

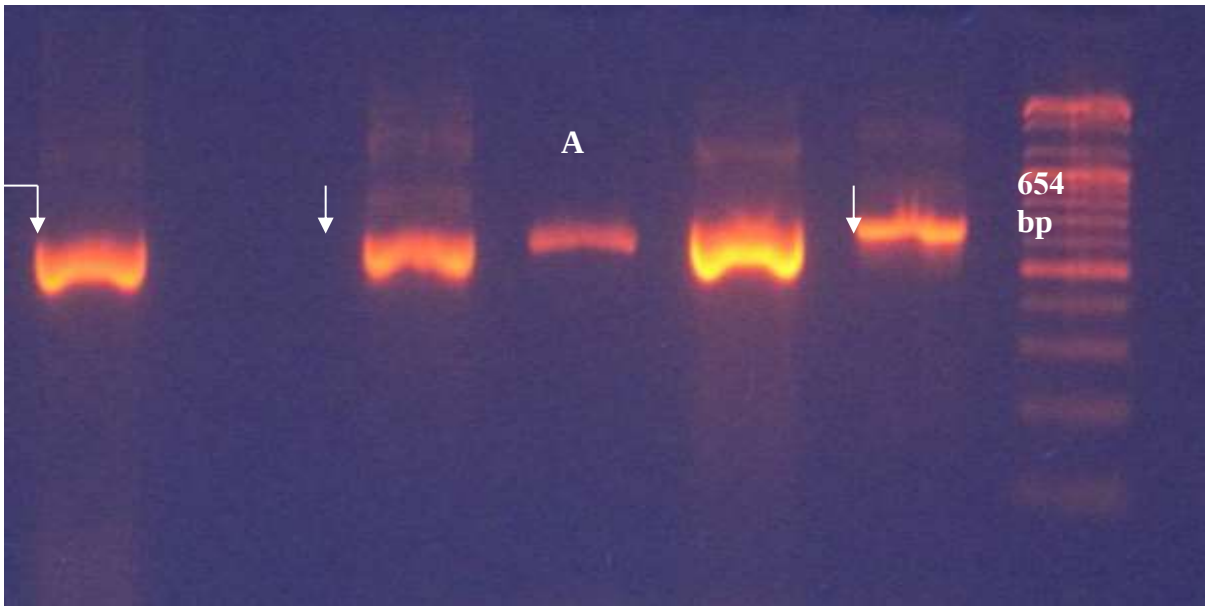

Figure (7) : PCR amplification of *nr* gene. M: Marker (100 bp DNA ladder) ;A: *E.cloacae* templates , B : *E.sakazakii* template.

D-Detection of Shiga Like Toxin gene

DNA templates of all *Enterobacter* spp (11 isolates). That isolated from stool specimens which were amplificated by PCR , they are not appear any bands of *slt* gene in expected size (215 bp) after were electrophoresised on agarose gel. The results for 11 of fecal culture were appeared, non of these 11 isolates involved *slt* gene. This may be due to not abundance of *slt* genes in these isolates , or because of either low a abundance of the SLT- producing organism in the sample or the *slt*- gene themselves being unstable that result from testing of individual isolates rather than primary fecal cultures in PCR assays [30] .

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