



Bacteriological study of *Enterobacter* spp. that isolated from different clinical specimens

Intidhaar Na'eem Abid

Dep. Pathological analysis –

College of science

Thi-Qar University

Assis.Prof Dr. Mahdi H.
Al-Amaar

Dep.Biology –College of science

Kufa University

Summary

The clinical specimens (718) were collected from inpatients and outpatients suffering from different infectious from (Al-Hussein, Maternity and Children, Surgical) hospitals and general laboratory health in Al-Nassiriyah city 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 (32%) among G- ve and (18%) from total isolates .It was distributed to 25(21%) from urine , 16 (19%) skin and soft tissues , 11(23%) stool , 11(17%) burn infections , 10 (22%) ENT , 5(28%) blood , 3(100%) CSF and 3(12%) post operations wounds ., and two species of *Enterobacter* , *E.cloacae* (89.3%) and *E.sakazakii* (10.7 %) were diagnosed . The virulence factors of bacteria were detected and 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.

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 (Patrson *et al.*, 2005). They produce acid upon glucose fermentation , methyl red negative , and Voges-Proskauer positive , with an optimal growth temperature 30° C, 80% are capsulated (Hart , 2006) , as well as certain biochemical properties , including the ability to



synthesize an enzyme known as ornithine decarboxylase , which is used to distinguish *Enterobacter* from the very similar and closely related *Klebsiella* bacteria .

Eterobacter spp. are considered opportunistic pathogen as other members of Enterobacteriaceae , these organisms produce significant virulence factors , such as endotoxins , that can mediate fatal infections , however , because they generally do not initiate disease in healthy , uncompromised human hosts , they are considered opportunistic (Forbes *et al* ., 2007). 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 (Grimont & Grimont , 2006 ; Farmer *et al.*, 2007).

Enterobacter spp. involved in extra intestinal infections are known to possess virulence-associated characteristics that distinguish them from random fecal isolates (Eisenstein & Jones , 1988) . Virulence factors are determine the initiation, development, and outcome of an infection involve a series of complex and shifting interaction between the host and the microorganism which can vary with different infecting microbes (Brodgen *et al* ., 2000) , these factors lies within cell structure or secretes are outside the body such as enzymes and toxins. These virulence factors such as haemolysin , capsule , protease, siderophore , colonization factor antigen (CFA) and bacteriocin(Hurst *et al.*,1996) . Because, the incidence of infection caused by *Enterobacter* spp. is increasing and littleness studies related to its ability to produce the virulence – associated properties, for these reasons, the aims of study was achieved by:

- 1 - Isolation and identification of *Enterobacter* species from different clinical specimens.
- 2- Detection some virulence factors such as haemolysin , capsule , protease , siderophore , colonization factor antigen (CFA) , bacteriocin.



Materials and Methods.

Identification of bacteria

A single colonies were isolated from primary positive cultures (on MacConkey , CHROMTM , EMB and XLD agar) and identified by morphological , biochemical tests and API 20 E(BioMerieux , France) according to the criteria of (Holt *et al.*, 1994; Collee, *et. al.*, 1996; MacFaddin ,2000) .

Detection of virulence factors

1.Hemolysin production was detection according to method of Baron et al. (1994).

2. Capsule stain

The capsule was stained according to method of Cruickshank *et al.* (1975)

3. Detection of protease production

By method of (Coque *et al.*, 1995; Elsner *et al.*, 2000).

4.Colonization Factor Antigen (CFA)

(Ofek *et al.*, 1977).

5. Siderophore production

The test was performed according the method of El-Sanousi *et al.* (1987) by transport a small amount of pure colony by sterile woody stick to M9 medium and the medium was incubated at 37° C for 24 hr., the results registered by depending on presence of bacterial growth on the medium .

6.Detection of bacteriocin production

Cup assay method was carried out (AL-Qassab & AL- Khafaji,1992).

Results

Distribution of *Enterobacter* isolates



Eighty four isolates of *Enterobacter* spp were isolated from 718 specimens that were collected from different infections during the period from February 2011 to June 2012 , were cultured on different media such as MacConkey agar , CHROMTM , EMB , XLD agar , the identification of bacterial isolates were depending on morphological , biochemical tests and API 20E kit, represented by : 25 (21%) isolates from urine , 16(19%) from skin and soft tissue ,11(23%) from stool , 11(17%) from burns , 10(22%) from ENT, 5(28%) from blood , 3(100%) from CSF and 3 (12%) from post surgical wounds (Table 1).

Table (1): Numbers of *Enterobacter* isolates from different clinical specimens

Type of specimens	No. of specimens	No.&(%) of negative specimens	No.&(%) of positive specimens	No.&(%) of G ⁻ bacteria	No.&(%) of G ⁺ bacteria	No.of <i>Enterob act-er</i> isolates	(%) of <i>Enterob act-er</i> isolates among G ⁻	(%) of <i>Enterob act-er</i> isolates among positive specimens
Urine	225	107(48%)	118(52%)	77(65%)	41(35%)	25	32%	21%
Skin & soft tissues	114	30(26%)	84(74%)	26(31%)	58(69%)	16	62%	19 %
Stool	50	2(4%)	48(96%)	46(96%)	2(4%)	11	24%	23%
Burns	81	15(19%)	66(81%)	53(80%)	13(20%)	11	21%	17%
ENT	58	%) 12(21	46(79%)	26(57%)	20(43%)	10	38%	22%
Blood	50	32(64%)	18(36%)	6(33%)	12(67%)	5	83%	28%
CSF	40	37(92.5)	3(7.5%)	3(100%)	0(0%)	3	100%	100%
Postsurgical wounds	40	(37.5%) 15	(62.5%) 25	14(56%)	11(44%)	3	21%	12%
Sputum	60	8(13%)	52(87%)	12(23%)	40(77%)	0	0%	0%
Total	718	258(36%)	460(64%)	263(57%)	197(43%)	84	32%	18%



These isolates contained two species: *E.cloacae* in the first order which registered 75 isolates (89.3%), and *E.sakazakii* (9) isolates (10.7%) (Table 2).

Table (2): Distribution of *Enterobacter* species

<i>Enterobacter</i> species	No. of isolates	%
<i>E.cloacae</i>	75	89.3
<i>E.sakazakii</i>	9	10.7
Total	84	100

The distribution of bacterial isolates on clinical specimens, was appeared that the *E.cloacae* was registered higher rate of its in urine sample (100%), followed by skin and soft tissues (87.5%), while it don't appeared in sputum specimens (Table 3).

Table (3): Distribution of *Enterobacter* species on clinical specimens

Type of specimens	No.&(%) of <i>Enterobacter</i> species		Total (%)
	<i>E.cloacae</i>	<i>E.sakazakii</i>	
Urine	25(100%)	0 (0%)	25 (11.1%)
Skin & soft tissues	14 (87.5%)	2 (12.5%)	16 (14%)
Stool	7(63.6%)	4 (36.4%)	11(22%)
Burns	8(72.7%)	3 (27.3%)	11(13.5%)
ENT	10(100%)	0 (0%)	10(17.2%)
Blood	5(100%)	0 (0%)	5 (10%)
CSF	3(100%)	0 (0%)	3 (7.5%)
Post surgical wounds	3(100%)	0 (0%)	3(7.5%)
Sputum	0(0%)	0 (0%)	0(0%)
Total	75(89.3%)	9 (10.7%)	84 (100%)

Virulence factors of *Enterobacter* species



The results of virulence factors of *Enterobacter* isolates, show that all *Enterobacter* didn't produced any type of hemolysin (table 4) .

All isolates of *Enterobacter* species had apolysaccharide capsule surrounding the bacterial cell except one isolate of *E.cloacae* that lack capsule structure (98.8% versus 1.2%).

From the results, it was appeared two species of *Enterobacter* isolates doesn't produce extracellular protease. On the other hand, all isolates of *Enterobacter* have the ability to produce siderophore 84 (100%).

Table(4): Virulence factors of *Enterobacter* species

Virulence factors	<i>E.cloacae</i> n= 75		<i>E.sakazakii</i> n=9		No. & (%) of all positive isolates	No. & (%) of all negative isolates
	No.& (%)of positive isolates	No.& (%)of negative isolates	No.& (%)of positive isolates	No.& (%)of negative isolates		
Hemolysin	0 (0%)	75 (100%)	0 (0%)	9(100%)	0 (0%)	84(100%)
Capsule	74(98.7%)	1(1.3%)	9(100%)	0 (0%)	83(98.8%)	1(1.2%)
Extracellular Protease	0 (0%)	75(100%)	0 (0%)	9(100%)	0 (0%)	84(100%)
Siderophore	75(100%)	0 (0%)	9(100%)	0(0%)	84(100%)	0 (0%)



Table (5) shows 32% of isolates were had ability to produce CFA / I , while the ability of isolates for produce CFA /II were less (10 %) , whereas *E. Sakazakii* isolates were don't produced this factor .

The high production of CFA, was appeared in produce of CFA / III, (95%) of *Enterobacter* isolates were production this factor.

Table (5): Colonization Factor Antigens of *Enterobacter* species

Virulence factors		<i>E.cloacae</i> N= 75		<i>E.sakazakii</i> n=9		No. & (%) of all positive isolates	No. & (%) of all negative isolates
		No.& (%)of positive isolates	No.& (%)of negative isolates	No.& (%)of positive isolates	No.& (%)of negative isolates		
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/II I	71(95%)	4(5%)	9(100%)	0(0%)	80(95%)	4(5%)

The Bacteriocin production was investigated using two type of indicator bacteria: *E.coli* and *Klebsiella pneumoniae* , in related of use *E.coli* as indicator, it was founded, 15 isolates (17.9%) of total bacterial isolates 84 of *Enterobacter* were able to produce bacteriocin , were 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*(Table 6).



Table (6): Bacteriocin production of *Enterobacter* species

Virulence factors		<i>E.cloacae</i> n= 75		<i>E.sakazakii</i> n=9		No. & (%) of all positive isolates	No. & (%) of all negative isolates
		No.& (%)of positive isolates	No.& (%)of negative isolates	No.& (%)of positive isolates	No.& (%)of negative isolates		
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*

Discussion

Enterobacter spp isolation and identification

recent study were included isolation of *Enterobacter* spp. from different of clinical specimens (urine, skin and soft tissues infection , stool , burns, CSF , post surgical wounds and sputum) , the result were appeared that , the total infections rate of *Enterobacter* spp. was 18% from total positive specimens and 32% from total G⁻ bacteria.

These results are similar to those reports of NNIS (1997) that demonstrated, *Enterobacter* species were caused 11.2% of infections in all types of ICUs , the corresponding rates among patients in pediatric ICUs were 6.8% for blood stream infections and 9.5% for UTIs. Recent study was registered high rate of *E.cloacae* in clinical specimens than in *E.sakazakii* , this relatively high prevalence of *E.cloacae* may be result from widely distribution in the environment , this bacteria occurs as a saprophyte in water , sewages ,



soil , meat , skin and copmmensal in the intestinal tracts of humans and animals (Dudley *et al.*, 1980 ; Eisenstein & Jones , 1988 ; Sommers , 1985) . It was appeared from our results *E.cloacae* was registered higher rate in urine specimens, this may be resulted from presence this bacteria in the normal flora of the human gastrointestinal tract and transferred to infected urinary tract (Grimont & Grimont, 2006). Pead & Maskell (1977) found the most of UTIs were resulted from bacteria that present in intestinal tract. *E.cloacae* also was appeared in high rate in skin & soft tissue infections , the source of these infection may be result from colonization of the skin by this bacteria as normal flora and caused endogenous infection (Fraser *et al.* , 2010) .

Oral and intestinal colonization with *E.sakazakii* may be associated with ingestion of contaminated food (Galili *et al.*,1995 ; O' Hara & Miller , 2003) .

E.sakazakii is an important emerging neonatal pathogen , associated with outbreaks of necrotizing enterocolitis (NEC) (Mullane *et al.*, 2007) , it is prevalent in certain milk - based powdered infant formula , cereals , chocolate , potato flour (Biering *et al.*, 1989) .

In relation of virulence factors of these bacteria. The results of recent study, show that all isolates of *Enterobacter* (*E .cloacae* and *E.sakazakii*) did not produce haemolysin (γ - hemolytic) . These results are in agreement with Keller *et al.*,(1998) who found non of 57 *Enterobacter* spp. tested were hemolytic. The results shown that most *Enterobacter* isolates had a capsule surround the bacterial cell. These results are partial agreement with those obtained by Salman (2006) who found all isolates of *E. cloacae* that isolated from UTIs had a capsule.

Protease is one of virulence factors of many microorganisms, it assist the hydrolysis of large polypeptides cell (Beynom & Bond ,1989). The study results revealed that , all isolates of *Enterobacter* were negative for this factor , these results are partial agreement with results obtained by Salman (2006) who found non of 9 isolates of *E.cloacae* have the ability to produce extra cellular protease. 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). These results are identical with study results of Clegg & Gerlach (1987) whom were isolated this factor from *Enterobacter* spp. this factor is responsible for colonization the surfaces of indwelling devices (e.g. urinary catheters) (Mobley *et al.*, 1988)

CFA/ I also was found in isolates of *Enterobacter* but in percentage less than that in CFA/ III ; Adegbola & Old (1983) ; Keller *et al.*(1998) they found mannose – sensitive hemagglutinin (MSHA) or type 1 fimbriae in most strains of *E.cloacae*.

The relationship between this factor and pathogenicity of bacteria was established from adherence of bacteria in mucous surfaces or epithelial cells of genital, urinary , respiratory and gastric tract (Venegas *et al.*,1995) . It was appeared that the lower presence of colonization factor antigens among isolates was CFA/II .This factor causes agglutination of chicken blood, and act to adhere of bacteria with specific and complex carbohydrate receptors of epithelial cells of small intestine (Ram *et al.*, 1995) .

As shown in the results all *Enterobacter* isolates are able to produce siderophore . Der Vartanian *et al.* (1992) 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 . These results are partial agreement with those obtained by Keller *et al.*(1998) that found 72.2% of *E.cloacae* that are able to produce siderophore.

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 (Riley & Chavan 2007).

Cup assay method was used for detection of bacteriocin production from *Enterobacter* isolates against other bacteria types which is *E.coli* and *Klebsiella pneumoniae* (sensitive bacteria) . 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 (VanTiel-Menkveld *et al.*, 1982) such receptor for the uptake siderophore also functions as receptor for the bacteriocin (cloacin)(Bouchet *et al.* , 1994).

The results were appeared , *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 used for typing are different in nature (Bauernfeind *et al.*, 1981) . These results are identical with (Traub *et al.*(1982) ; Salman (2006)) they found *Enterobacter* bacteriocin was inactive against *Klebsiella pneumoniae* , but these results are disagreement with results of Riley *et al.*(2003) that the activity of *Enterobacter* bacteriocin was affected not only *Enterobacter* spp. , but also other related bacteria such as *Klebsiella pneumoniae* .

دراسة بكتيريولوجية لبكتريا *Enterobacter* spp. المعزولة من عينات مرضية مختلفة

ا. م. د. مهدي حسين العمار
قسم علوم الحياة – كلية العلوم
جامعة الكوفة

انتظار نعيم عبد
قسم التحليلات المرضية - كلية العلوم
جامعة ذي قار

الخلاصة

شملت الدراسة جمع 718 عينة مختلفة من المرضى الراقدين والوافدين الذين يعانون من إصابات مرضية مختلفة لكل من مستشفيات (الحسين التعليمي ، الولادة والأطفال ، الجراحي العام) ومختبر الصحة العام في مدينة الناصرية خلال الفترة من شباط لسنة 2011 إلى حزيران لسنة 2012. شخّصت عزلات بكتريا *Enterobacter* باستخدام الفحوصات المظهرية والبايوكيمياوية إضافة إلى نظام E API 20. تم تشخيص 84 عزلة لبكتريا *Enterobacter* spp. شكلت نسبة 32% بين البكتريا السالبة لصبغة كرام و 18% من بين جميع العزلات البكتيرية (السالبة والموجبة لصبغة كرام) ، توزعت إلى 25 (21%) من الإدرار ، 16 (19%) من الجلد والأنسجة الرخوة ، 11 (23%) من البراز ، 11 (17%) من الحروق ، 10 (22%) من مسحات الأذن والأنف والحنجرة ، 5 (28%) من عينات الدم ، 3 (100%) من سائل النخاع الشوكي و 3 (12%) من مسحات جروح العمليات الجراحية. تم عزل نوعين من بكتريا *Enterobacter* spp. من كل العينات المرضية المشمولة بالدراسة ، تضمنت بكتريا *E. cloacae* و *E. sakazakii*. بينت الدراسة أن النوع *E. cloacae* هو الأكثر شيوعا في الإصابات المرضية المتسببة عن هذا الجنس ، إذ تم الحصول على 75 عزلة (89.3%) تعود لهذا النوع ويليها النوع *E. sakazakii* إذ عزل بواقع 9 عزلات (10.7%) . درست بعض عوامل الضراوة لهذه البكتريا وقد ظهر أن جميع العزلات كانت حاوية على المحفظة وتمتلك نظاما واحدا فقط للحصول على الحديد بقابليتها على إنتاج السايدروفور (siderophore) وعدم قابلية جميع العزلات على إنتاج الهيمولايسين (haemolysin) و إنتاج إنزيمات البروتيز الخارجية . أظهرت النتائج أن نسبة عالية من العزلات (95%) امتلكت عامل الاستيطان الثالث CFA / III و 15 (17.9%) من مجموع العزلات لها القابلية على إنتاج البكتريوسين .

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