Identification and Characterization of *Raoultella* (formerly *Klebsiella*) species Isolated from Clinical Samples in Hilla

A thesis

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تشخيص وتوصيف انواع بكتريا الراولتيلا (الكليبسيلا سابقا) المعزولة من عينات سريرية في الحلة

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مجلس كلية الطب في جامعة بابل كجزء من متطلبات نيل درجة

الماجستير في علم الأحياء المجهرية الطبية

من قبل

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هـ

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ ۞ خَلَقَ الإنسان مِنْ عَلَقٍ ۞ اقْرَأْ وَرَبُّكَ الْأَكْرَمُ ۞ الَّذِي عَلَّمَ بِالْقَلَمِ ۞ عَلَّمَ الإنسان مَا لَمْ يَعْلَمْ)

صدق الله العظيم

سورة العَلَق (الآية 1-5)

Dedication

To …..

Who support me: My Father

The kind heart: My Mother

Who supply me: My Brothers

Samah & Ali

I dedicate this work.

Samir

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Praise to the mighty God, the glorious creator of the universe, the engineer of the life, for his kindness and mercy, and blessings upon Mohammed the prophet and upon his family.

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I wish to thank the following hospitals in Babylon province for providing clinical samples: (Teaching Hospital of Hilla, Mergan Hospital, and Maternity and Pediatric Hospital) .

Supervision Certificate

We certify that this thesis under subject **(Identification and Characterization of *Raoultella* (formerly *Klebsiella*) species Isolated from Clinical samples in Hilla)** was prepared under our supervision at the Department of College of Medicine/Babylon University, as a partial requirements for the degree of **Master of Science in Medical Microbiology (Bacteriology)** and this work has never been published anywhere.

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Summary :

During a four-months period, a total of 720 clinical samples were collected from three main hospitals in Hilla-city Babylon province. Sample were screened for the presence of *Raoultella* spp., as well as studying their expression of virulence factors.

A total of 144 bacterial isolates were recovered and identified as *Klebsiella*-like organisms. Out of these, 11 isolates were identified as *Raoultella*, which represent 7.6% of all *Klebsiella*-like organisms found. These isolates were further identified as *R. ornithinolytica*. *R. planticola* and *R. terrigina* were not isolated in this study. Many virulence factors expressed by *R. ornithinolytica* were studied in vitro. All isolates (100%) produced capsule and expressed CFA/I, and CFA/III.

Nine isolates (81.8%) were able to produce siderophores. Four isolates (36.6%) were able to produce bacteriocin. All *R. ornithinolytica* isolates were unable to produce extracellular protease, hemolysin, and histamine.

All isolates of *R. ornithinolytica* (100%) were resistant to penicillin, ampicillin, gentamicin, chloramphenicol, rifampin, cephalothin, cephotaxime, streptomycin, amoxicillin.Ten isolates (90.9%) were resistant to tetracycline, and (63.6%) 7 isolates, were resistant to naldixic acid. On the other hand, *R. ornithinolytica* expresses a low degree of resistance to nitrofurantoin and ciprofloxacin (18.1%), and all isolates(100%)

were sensitive to meropenem. These drugs may be considered as drugs of choice for the treatment of infections caused by *R. ornithinolytica*.

R. ornithinolytica expressed a high degree of sensitivity to the effect of human serum when they grew in human serum at 37 ^{o}C for 3 hrs .

The present study represented the first investigation of the occurrence of *Raoultella* spp. in human clinical sample, and the first record of occurrence of *R. ornithinolytica* in human clinical samples in Iraq.

الخلاصة :

هدفت هذه الدراسة الى الكشف عن وجود بكتريا *Raoultella* في العزلات السريرية وكذلك دراسة عوامل الضراوة فيها . جمعت في هذه الدراسة 720 عينة سريرية من ثلاث مستشفيات رئيسية في مدينة الحلة – محافظة بابل ,هي (مستشفى الحلة التعليمي ، مستشفى مرجان , ومستشفى الولادة والاطفال) خلال المدة من تشرين الثاني2006 ولغاية اذار 2007. شخصت حوالي 144 عزلة بكتيرية كـ (*Klebsiella* – like organisms) .

اظهرت هذه الدراسة عائدية11 عزلة من هذه العزلات الى *Raoultella* spp. والتي تمثل 7.6% من كل (*Klebsiella*-like organisms). جميع العزلات من *Raoultella* شخصت كـ *R. ornithinolytica* .

لم تعزل في هذه الدراسة الانواع *R. planticola* و *R. terrigena* العائدة لنفس الجنس.

اظهرت النتائج ان جميع عزلات *R. ornithinolytica* قادرة على التعبيرعن العديد من عوامل الضراوة كإنتاج الكبسولة وإنتاج عوامل الاستيطان الثلاثة (عامل الاستيطان الاول والثالث).تسع عزلات من *R. ornithinolytica* (81.8%) قادرة على انتاج السدروفور.

كما اظهرت النتائج قابلية اربعة عزلات من *R. ornithinolytica* (36.6%) على انتاج البكتريوسين . غيران جميع هذه العزلات كانت غير قادرة على انتاج البرواتيزات الخارجية والهيمولايسين والهستامين.

كانت جميع عزلات *R. ornithinolytica* مقاومة للبنسلين ,الامبسلين,الجنتمايسين ,الكلورمفينيكول ,الرفامبين ,السيفالوثين ,السيفوتاكزايم والنالدكسك اسد(63.6%). ان عزلات *R.ornithinolytica* تظهر درجة واطئة من ألمقاومة لكل من النايتروفيرتيون والسبروفلاكسيسين(18.1%) وان جميع العزلات كانت تعتبر حساسة لمضاد الميروبنيم.

ان حساسية عزلات *R. ornithinolytica* لمضادات النايتروفيرتيون، السبروفلاكسيسين و الميروبنيم قد جعلت هذه المضادات هي المختارة لمعالجة الاصابات التي تسببها *R. ornithinolytica*.كما اظهرت النتائج ان بكتريا *R. ornithinolytica* حساسة للمصل البشري عند تنميتها فيه لمدة ثلاث ساعات بدرجة 37 درجة سيليزية.

ان هذه الدراسة تعد الاولى للكشف عن تواجد عزلات *Raoultella* في العينات البشرية السريرية والتسجيل الاول لتواجد بكتريا *R. ornithinolytica* في العينات البشرية السريرية في العراق.

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List of Abbreviations

Abbreviation	Key
β-lactam	Beta-lactam
bla gene	Beta lactamase gene
C3b	Complement component 3 b
C4b	Complement component 4 b
C5	Complement component 5
C5b-C9	Complement component 5- Complement component 9
C1q	Complement component 1 q
CFA/I	Colonization factor antigen I
CFA/II	Colonization factor antigen II
CFA/III	Colonization factor antigen III
DNA	Deoxy ribonucleic acid
ETEC	Enterotoxogenic *Escherichia coli*
HPB	Histamine producing bacteria
HDC	Histidine decarboxylase
hdc gene	histidine decarboxylase gene
HFP	Histidine fish poisoning
ICU	Intensive care unit
IU	International unit
KDa	Kilodalton
LPS	Lipopolysacchrides
MRHA	Mannose resistant hemmagglutination

Abbreviation	Key
MR/KHA	Mannose resistant and *Klebsiella* like hemmagglutination
MrkD polypeptide	Mannose resistant *Klebsiella* (D) polypeptide
MSHA	Mannose sensitive hemagglutination
MAC	Membrane attack complex
NCCLS	National Committee of Clinical Laboratory Standards
ORN-1 gene	*ornithinolytica*-1 gene
PBP_S	Pencillin binding proteins
PCFs	Putative colonization factors
PLA-1 gene	*planticola*-1 gene
16S rRNA	16 stands for sevedberg unit ribosomal ribonuclic acid
rpoB	RNA polymerase β-subunit
R-LPS	Rough-lipopolysacchrides
rpm	revolution per minute
rRNA	Ribosomal ribonuclic acid
S-LPS	Smooth-lipopolysacchrides
tRNA	Transfer ribonuclic acid
TEM- 1 gene	Temonera–1 gene
UTI	Urinary tract infection

Conclusions:

1. *Raoultella ornithinolytica* was the predominant species (100%) among *Raoultella* spp. in Hilla-city/Babylon province.
2. *Raoultella ornithinolytica* isolates observed in the present study represented (7.6%) in human clinical sample which indicate the presence of a cryptic bacteria at a high frequency in human sample, and this disproves the previous idea that indicates the presence of *Raoultella ornithinolytica* is limited in the environment.
3. The expression of several virulence factors by *Raoultella ornithinolytica* isolates indicating their ability to cause different infections in human.
4. *Raoultella ornithinolytica* have a high percentage of resistance for many antibiotics such as β-lactam and other non - β-lactam antibiotics.
5. Meropeneme, ciprofloxacin, and nitrofurantion, may be considered as drugs of choice for treatment of infections caused by *R. ornithinolytica* isolates.
6. *Raoultella ornithinolytica* seems to be sensitive to serum activity.

Recommendations:

1. Study the occurrence of *Raoultella* spp. in human clinical samples in other parts of Iraq.
2. Study the ability of these bacteria to colonize human tissues.

3-Apply the immunological studies to enhance the acquired immune response against *Raoultella* spp. especially in immunocompromised state and in age extremes individuals.

Appendix (1): *Raoultella.ornithinolytica* isolated during the study

Isolate	Sample	Place of study	Sex	Age
R.ornithinolytica 13	Stool	Maternity and Pediatrics hospital	Female	(8) months
R.ornithinolytica 26	Stool	Maternity and Hediatrics hospital	Female	(8) months
R.ornithinolytica 27	Stool	Teaching hospital of Hilla	Female	(4) years
R.ornithinolytica 32	Stool	Maternity and Pediatrics hospital	Female	(4) years
R.ornithinolytica 45	Stool	Teaching hospital of Hilla	Female	(1.7) years
*R.ornithinolytica*70	Stool	Maternity and Pediatrics hospital	Male	(3) years
R.ornithinolytica 99	Stool	Teaching hospital of Hilla	Male	(3) years
R.ornithinolytica 163	Stool	Maternity and Pediatrics hospital	Male	(20) years
R.ornithinolytica 104	Urine	Maternity and Pediatrics hospital	Female	(25) years
R.ornithinolytica 398	Urine	Maternity and Pediatrics hospital	Male	(2) years
R.ornithinolytica 487	Urine	Maternity and Pediatrics hospital	Male	(3) years

Appendix (2) :Antibiotic resistance of *Raoultella ornithinolytica* to antibiotics

Bacterial isolate	Resistant (+) to antibiotic													
	P	Amp	Amx	KF	CTX	MEM	GN	S	RD	TE	F	NA	C	CIP
*R.ornithinolytica*13	+	+	+	+	+	-	+	+	+	+	+	+	+	+
R.ornithinolytica 26	+	+	+	+	+	-	+	+	+	+	-	-	+	+
R.ornithinolytica 27	+	+	+	+	+	-	+	+	+	+	+	-	+	+
R.ornithinolytica 32	+	+	+	+	+	-	+	+	+	+	+	+	+	+
R.ornithinolytica 45	+	+	+	+	+	-	+	+	+	-	+	-	+	+
R.ornithinolytica 70	+	+	+	+	+	-	+	+	+	+	+	+	+	+
R.ornithinolytica 99	+	+	+	+	+	-	+	+	+	+	+	-	+	+
R.ornithinolytica 104	+	+	+	+	+		+	+	+	+	+	+	+	-
R.ornithinolytica 163	+	+	+	+	+	-	+	+	+	+	+	+	+	-
R.ornithinolytica 398	+	+	+	+	+	-	+	+	+	+	+	-	+	+
*R.ornithinolytica*487	+	+	+	+	+	-	+	+	+	+	+	-	+	+

Chapter One

Introduction
and
Literatures Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Conclusions

and

Recommendations

References

Appendices

1-1 Introduction:

The genus *Raoultella* is a member of the family Enterobactericeae. This genus includes three species *R. ornithinolytica*, *R. planticola* and *R. terrigna*. Originally, *Raoultella* spp. though to occur solely in aquatic, botanic, and soil environments and has been never been isolated from clinical specimens (Gavani *et al*., 1977; Bagley *et al.,*1978; Bagley *et al.,* 1981; Izard *et al.,* 1981; Sakazaki *et al.,* 1989).

The first isolation of *R.planticola* from neonates in neonatal wards reported by Podschun *et al*.,(1998).Monnet and his colleagues (1991) mentioned that *R.planticola* may found in human clinical specimens and represent 8% of clinical isolates, while, *R. terrgina* is rarely found, which represent as 0.4% among clinical *Klebsiella* strains (Podschun *et al*., 1992).

Recent studies showed that *Raoultella* isolated from clinical specimens has many virulence factors such as capsule, colonization factors antigens (CFA/I and CFA/III), production of siderophore, histamine and bacteriocin (Podschun *et al.,* 1993; Podschun and Ullmann, 1998; Kanki *et al.,*2007).

It was also found that *R. planticola* strains were resistant to azteornam, nitrofurantoin, penicillins, and gentamicin but suscebtiple to piperacillin, imipenem, and ciprofloxacin (Liu *et al*., 1997; Podschun *et al.,*1998).

The aims of this study are to investigate the occurrence of *Raoultella* spp. isolates in human clinical sample in Hilla-city, characterizing the isolates and exploring some of the common virulence factors that contribute to the pathogensis, ability of *Raoultella* spp. isolates to express some virulence factors, and evaluating the susceptiblity of isolates to some antibiotics.

1-2 Literatures Review:

1-2-1: Taxonomy of *Raoultella* spp:

In the early of 1980s, Gavini and his colleagues (1977) classified environmental *Klebsiella* strains into provisional taxa groups J, K, L, and M (*Klebsiella*-like organisms) from which three new species have imerged *Klebsiella terrigena, K. planticola*, and *K. ornithinolytica*.

In 1981, two new environmental species were described, *Klebsiella planticola* and *Klebsiela terrigena,* on the basis of DNA-DNA hyberdization which have been found in soil and surface water (Bagely *et al.,* 1981; Izard *et al.,* 1981).

Qraskov and Qraskov (1984) classified envirometal *Klebsiella* into *Klebsiella planticola* and *Klebsiella terrigena*. On the same manner, Sakazaki *et al.*(1989) described *Klebsiella ornithinolytica* and showed that it was related to botanic environments. Ferragut *et al.*(1983) described that *K. trevasanii* was isolated from water and soil. *K. planticola* and *K. trevasanii* combined into one species, *K. planticola* because of their extensive DNA sequence homology (Gavini *et al.,* 1986).

More recently, in 2001, phylogentic analyses of *Klebsiella* spp confirmed that the genus *Klebsiella* is heterogeneous and composed of species which form three clusters that also included members of other genera as follows:

Cluster 1 includes the type strains of *Klebsiella pneumoniae* subsp. *pneumonia, K. pneumoniae* subsp.

rhinosecleromatis, and *K. pneumoniae* subsp. *ozaneae*; cluster 2 includes *K. ornithinolytica, K. planticola*, and *K. terregina* organisms characterized by ability to grow at 10ºC and utilization of L-sorbose as a carbon source; and cluster 3 includes *K. oxytoca.*

The data from sequence analyses (16SrRNA and rpoB genes) along with the previously reported biochemical and DNA-DNA hybridization data support the division of the genus *Klebsiella* into two genera. The name *Raoultella* is proposed as a genus name of species of cluster 2 by Drancourt *et al.* (2001) in France.

1-2-2 Occurrence of *Raoultella* spp:

Raoultella spp. though to occur solely in a aquatic, botanic, and soil environments in France (Gavini *et al.,* 1977). Two new environmental species, *K. planticola* and *K. terrigena,* have been found in soil and surface water in Oregon and Japan, respectively (Bagely *et al.,* 1981; Izard *et al.,* 1981). Sakazaki *et al.*(1989) found that *K. ornithinolytica* isolates were related to botanic and aquatic environments in Japan. Several studies reported that *K. terrigena* and *K. planticola* were isolated from clinical specimens (Podschun & Ulmmann 1994; Monnet & Freney 1994; Podschun *et al.,* 1998; Toivanen *et al.,* 1999; Westebrook *et al.,* 2000).

R. planticola and *R. ornithinolytica* strains were also isolated from fish as a histamine-producing bacteria (Kanki *et al.,* 2002 ; Kanki *et al.,* 2007).

1-2-3 General characteristics of *Raoultella* spp:

The genus of *Raoultella* is a member of the family Enterobactoraceae that is composed of gram-negative, oxidase-negative, catalase positive, aerobic or facultatively anaerobe, non motile, encapsulated rods, and having both a respiratory and a fermentative type of metabolism (Drancourt *et al.,* 2001).The bacterial cells grow on meat extract medium, giving dome shape and glistering colonies, varying in degree of stickiness, grow in 37°C and able to grow at 10°C (Sakazaki *et al.,*1989; Drancourt *et al*., 2001). Most strains can use citrate and glucose as a sole carbon source. Glucose is fermented with production of acids and gases, and most strains produce 2,3-butanediol as a major end product of glucose fermentation, Vogas-Proskauer test always positive (Table 1-1), also this table shows that the differential tests between *Klebsiella* and *Raoultella* spp. There are three types of *Raoultella* spp: *Raoultella planticola*, *Raoultella terrigena* and *Raoultella ornithinolytica* (Drancourt *et al.,* 2001).

Table (1-1) : Differentiation of *Klebsiella* and *Raoultella* species

Test	*Klebsiella* spp.				*Raoultella* spp.		
	K.oxytoca	*K.pneumoniae* subsp. *ozaenae*	*K.pneumoniae* subsp. *pneumoniae*	*K. pneumoniae* subsp. *rhinoscleroma*	*R.ornithinolytica*	*R. planticola*	*R.terrigena*
Gram stairn	-	-	-	-	-	-	-
Growth at 10 ºC	+	-	-	-	+	+	+
H_2S production	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-
Acid from:							
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Sucrose	+	-	+	+	+	+	+
L-arabinose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	
D-Mannitol	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+
Sorbose	+	-	-	-	+	+	+
D-Gluccoseamine	+	-	+	+	-	+	+
IMViC test							
Indole production	+	-	-	-	+	+	-
Methyl red	+	-	+	+	+	+	+
Vogas - Proskaur	+	-	+	-	+	+	+
Citrate	+	+	+	-	+	+	+
Histamine	-	-	-	-	-	+	+
Ornithine decarboxylase	-	-	-	-	+	-	-
Urease	+	-	+	-	+	+	+
Gelatinase (protease)	-	-	-	-	-	-	-
L-Tartrate	+	+	-	+	-	-	-

From : (Drancourt *et al.,* 2001; Hansen *et al.,* 2004; Alves *et al.,* 2006).

1-2-4 *Raoultella* as a pathogenic organism:

Raoultella spp. in early researches is an environmental bacteria that occurs in soil, botanic and aquatic environments (Gavini *et al.*, 1977; Bagley *et al.,* 1978; Bagley *et al.*,1981; Izard *et al.*, 1981; Sakazaki *et al.*, 1989; Podschun *et al.,* 1992).

Monnet and Freney (1994) reported that *R. planticola* and *R. terrigena* have been isolated from clinical specimens and has been associated with various infections including septicemia. They also found that *R. planticola* may represent 8-19% of clinical *Klebsiella* isolates and considered them as opportunistic pathogens.

The first report on the isolation of *R. planticola* from neonates in intensive care unit and their ability to colonize pediatric patient which represent 9% of all *Klebsiella* spp. isolates was recorded by Podschun *et al.* (1998). Westebrook *et al*. (2000) found that members of the genus *Raoultella* are responsible for nasocomial infections in adult and for outbreaks in newborn populations in hospital.

Producing of virulence factors by clinical isolates of *R. planticola* is similar to virulence factor of *Klebsiella pneumoniae*, suggesting that they may have similar pathogencity (Podschun *et al.,* 2000). Also *R. planticola* and *R. ornithinolytica* isolated from fish has been reported as a histamine producing bacteria (Kanki *et al.,* 2002; Kanki *et al.*, 2007).

Histamine fish poisoning caused by eating spoiled fish (Taylor *et al.,* 1989). The implicated fish are mainly of the family scomberesocidae which contain large amount of histamine that is produced by the microbial decarboxylatoin of the free hidstidine in the muscular tissue of fish (Taylor *et al.*, 1989).

1-2-5 Virulence factors of *Raoultella* spp:

Until 1998 nothing was known of the ability of *Raoultella* strains to express virulence factors thought to contribute to the pathogencity of *Raoultella* spp. (Podschun *et al.,* 1986; Podschun and Ullmann 1998). Podschun *et al.,* (2000) found that *Raoultella* express the same virulence factor of *K. pneumoniae.* The virulence factors of *Raoultella* spp. include capsular polysacchride, pili, siderophore, colonization factors production and serum resistance (Podschun *et al.,* 1986; Podschun *et al.,* 1993; Podschun and Ullmann 1998; Podschun *et al.,*2000).

1-2-5-1 Capsular polysacchrides:

Capsules are essential to virulence of *Raoultella* (Dominco *et al.,* 1982; Simmon-Smith, 1986; Drancourt *et al*., 2001). The capsular material forms thick bundle of fibrinous structures, this protects the bacterium from phagocytosis by polymorphonuclear

granulocytes, and prevents killing of the bacteria by bactericidial serum factors (Simmon-Smith,1986; Podschun *et al.,* 1992).

Capsular polysacchrides have been regarded as the main determinant of *Raoultella* pathogencity, and 77 different capsular types have been reported by Qraskov *et al .,* (1977). A number of other virulence factors have been described. In *K. pneumoniae*, the most frequent capsular serotype was K2 which represents 13.5% of all *Klebsiella* serotypes (Simmon-Smith, 1986). Other study showed that this K antigen to be very common among *Klebsiella* isolates. In *R. planticola* by contrast, K14 was predominant capsular type (13%). However, the frequency of *R. planticola* isolates expressing the K2 antigen (8.7 %) was similar to that in *K. pneumoniae* (Podschun, 1990). This sheds some light on the pathogenic capability of *Raoultella planticola*, as serotype K2 and K1 strains which are known to be such a virulent than other capsular types (Mizuta *et al.,* 1983; Nassif *et al.,* 1989).

Among the three most common *R. planticola* serotype, K70 was found as K2 (8.7%) capsular type. K70 appears to be closely associated with species. This serotype was not detected among 207 *Klebsiella* isolates investigated (Podschun and Ullmann, 1994).

1-2-5-2 Colonization factors of *Raoultella* spp (pili):

As a critical first step in the infections process, microorganisms must come as close as possible to host mucosal surfaces and maintain this proximity by attaching to the host cell adherence. Also The adhesive properties in the Enterobacteraceae are generally mediated by different types of pili which are non flagellar, filamentous projections on the bacterial surface (Jones *et al.,* 1983).

Pili are demonstrated mainly on the basis of the ability to agglutinate erythrocytes of different animal species. Depending on whether the reaction is inhibited by D-mannose, these adhesions are designated as mannose-sensitive or mannose–resistant hemagglutination, (MSHA and MR/KHA, respectively) (Ottow, 1975).

CFA/I are antigencally unrelated to the type1 pili generally associated with *E. coli* , and each is demonstrable by mannose–resistant hemmagglutination activity, whereas the type1pili mediate only mannose-sensitive hemagglutination (Evans *et al.,*1977; Qrskov and Fife, 1977).

The MSHA (type 1pili) was detected in 83% of *R. planticola* isolates in guinea Pig erythrocytes, whereas, MR/KHA was observed in 69% of these isolates and the incidences of MSHA and MR/KHA, serum resistance, fimbriae, and siderophore in *R. planticola* were very similar to those

found in *K. pneumoniae* (Podschun *et al.,* 2000). There are three types of colonization factors :

1-2-5-2-1 Colonization factor antigen/I (CFA/I): CFA/I is the best investigated tool of the bacterial adhesions, they are MRHA which agglutinate human erythrocytes group A in presence of mannose (Evans *et al.,* 1977; Evans and Evans 1978). The adhesion protein in pilus type is located on the fimbrial shaft and is capable of binding to mannose containing tri-saccharide of host glycoproteins (Firon *et al.,* 1984).

Maayan *et al*,. (1985) found that *Klebsiella pneumoniae* isolates possessed CFA/I.

The relevance of these pili to bacterial virulence is thought to arise mainly from binding of the bacterium to mucosa epithelial cells of urogential, respiratory and urinary tract infections (UTI). They studied in *E. coli* but described in *K. pneumoniae* in animal model (Ofek and Beachey, 1978). CFA/I are no longer of use to bacteria, since they trigger an opsonin-independent leukocyte activity known lectinophagocytosis (Ofek *et al*,.1995).

1-2-5-2-2 Colonization factor antigen/II (CFA/II): CFA/II mediate mannose-resistant hemmagglutination which agglutinate cow and chicken blood in presence of mannose (MRHA) (Evans *et al.,* 1977; Evans and Evans 1978). Studies

revealed that these antigens in enterotoxigenic *E. coli* mediate severe diarrhea (Evans and Evans 1978; Neeser *et al.,* 1989). CFA/II were also found in *K. pneumoniae* isolates (Duguid and Old, 1980; Ram *et al.,* 1995).

1-2-5-2-3 Colonization factor antigen /III(CFA/III): CFA/III mediate mannose-resistant and *Klebsiella* like hemmagglutination (MR/KHA). CFA/ III agglutinate only with human erythrocytes, that have been treated with tannic acid. In kidneys, these pili mediate bacterial adhesions to tubular basement membranes, Bomnan's capsules and renal vessels. CFA/III bind to tannic acid or heated-treated erythrocytes but not untreated erythrocytes (Trakkanen *et al.,* 1990 ;Trakkanen *et al.,* 1992; Podschun *et al.,* 1993).

The role of CFA/III in UTI are unknown, but the importance of this fimbrial type for persistence of bacteria in catheter-associated bacteruria has been demonstrated (Mobely *et al.,* 1988). Several types of colonization factor antigens (CFAs) and putative colonization factors (PCFs) have been identified on the basis of antigenic specificity and / or N-terminal amino acid sequences of the major subunit (pilin) eg. CFA/I ,CFA/II , CFA/III ,PCF (Nataro *et al .,* 1998).

1-2-5-3 Siderophore production:

Iron starvation is one of the major barriers that virulent bacteria must overcome in order to proliferate in the host. Virtually all microorganisms possess high affinity iron Fe^{3+} transport systems mediated by low molecular weight iron specific chelators called siderphores, the synthesis of which is iron-limiting condition (De Lorenzo and Martinez,1988).

There are two types of siderophors :

1-2-5-3-1 Phenolate-type siderophores: The most common group and their best known enterobactin representative,(also known as enterochelin), is a cyclic trimer of 2,3-dilydroxy-benzoyl-serine. This siderophore appears to comprise the main iron uptake systems of Enterobacteriaceae and is synthesized by almost all clinical isolates of *E. coli* and *Salmonella* spp. (Griffiths *et al.,* 1988).

1-2-5-3-2 Hydroxyamate-type sidrephores: the ferrichromes; which are synthesized only by fungi, the ferrioxamines, and aerobactin are most important. In contrast to enterobactin, the contribution of aerobactin to bacterial virulence has been clearly demonstracted (De Lorenzo and Martinez,1988). The observations of Martinze *et al.,* (1987) indicate that the enterobacterial genera can be divided into two groups according to their incidence of aerobactin synthesis. The group with a low rate of aerobactin producing strains (<20%) comprises genera such as *Serritia*, *Proteus* and *Salmonella*, the second group

which includes the genus *E. coli* shows a high incidence of aerobactin synthesis (>40%).

Recently, It was found that their are three types of siderophore systems for Enterobacteriaceae. Their most prevalent are: enterobactin, aerobactin, and yersiniabactin (Raymond *et al*., 2003**;** Mokracka *et al*., 2004).

Phenolate siderophore (yersiniabactin) is a siderophore system which first described in *Yersinia* species, but it can be found among some isolates of other enterobacterial species and is believed to be acquired via horizontal gene transfer (Bach *et al.,* 2000).

In *R. planticola* the production of both enterobactin and aerobactin has been demonstrated (Podschun *et al.,*2000). Enterobactin is synthesized by most strains of Enterobacteriaceae (William's *et al.,* 1987; Podschun *et al.,* 1992).

Aerobactin postive *Raoultella* isolates irrespective of the species or source of isolation have been observed rarely (Podschun *et al.,* 1992). Nassif and Sansonetti (1986) showed that an association between aerobactin synthesis and the virulence of *Raoultella* strains was unequivocally demonstrated.

Lawlor *et al.,* (2007) suggested that yersiniabactin contributes to a more virulent phenotype of *K. pneumoniae*, and an earlier study by Nassif and Sansonetti (1986) suggested that aerobactin can enhance the virulence levels of

less virulent strains. They together, suggested that the acquisition of a siderophore system in addition to the ubiquitous enterobactin system, whether it is yersiniabactin or aerobactin, may be a key factor in enhancing the virulence of *K. pneumoniae*.

1-2-5-4 Serum resistance of *Raoultella* spp:

Pathogenic microorganisms have developed strategy to counter the serum bactericidal effect; most commansal Gram-negative bacteria are sensitive to the bactericidal effect of human serum, whereas pathogenic strains often exhibit serum resistant properties (Olling, 1977).

The features "serum resistance" have been correlated with onset of infection, severity, and symptoms (Gower *et al.,* 1972). The main role of the serum bactericidal system is thought to prevent microorganisms from indwelling and persisting in the blood (Taylor, 1983).

Lipopolysaccharides (LPS) is generally able to activate complement, C3b is subsequently deposited onto the LPS molecules. However, since it is fixed preferentially to the longest O-polysaccharides chains, C3b is far away from bacterial cell membrane. Thus, the formation of lytic membrane attack complex (MAC) is presented, and subsequent membrane and cell death do not place, the quantity of deposited C3b that also determines the degree of serum resistance.

Bacterial molecules implicated in C3b deposited are the outer membrane porins proteins and smooth and rough lipopolysacchrides porins activate the classical pathway, causing deposition C3b on serum sensitive strains .

The smooth lipopolysacchrides (S-LPS) of serum resistant strains activates only the alternative pathway impeding the binding of C1q to porins and rough lipopolysacchrides(R-LPS) molecule and thereby preventing activation of the classical pathway. After its deposition, C3b is quickly degraded and it forms inactive C3b on both types of the strains, but the higher level deposition of C3b on serum-sensitive strains, resulting from activation of both the alternative and classical complement pathways, supports further complement activation and killing of serum-sensitive strains (Alberti *et al.,* 1996).

1-2-5-5 Histamine Production:

Raoultella posses the *hdc* gene, that encoding pyridoxal phosphate-dependent histidine decarboxylase that breaks histidine leading to formation of histamine (Guired and Srell, 1987; Kamath *et al.,*1991; Wauters *et al.,*2004). Histamine fish-producing bacteria, *Raoultella planticola* and *Raoultella ornithinolytica* posses histidine decarboxycase (HDC), which converts histidine in muscular tissue into histamine.

Histamine fish poisoning attributes to ingestion of Scombroid fish (family Scomberesocidae) containing high

levels of histamine between (2.810-5.250 mg/L) produced by histamine producing bacteria (HPB).

Freezing greatly decreased the histamine producing ability of HPB. Especially in *Photobacterium phosphorm* it has been speculated that HFP is caused by histidine decarboxylase itself from HPB cells autolysing during frozen storage, even when HPB survives frozen storage to estimate bacteria that produce histamine with various temperature, pH levels and NaCl concentrating (Kanki *et al.,* 2007).

Histamine fish poisoning is responsible for cardiac and respiratory manifestation (Lehane and Olley, 2000). According to the results of studies mentioned above, Histamine production by *Raoultella* spp. could be considered as a virulence factor in pathogencity of these bacteria in food posioing comparing with non histamine production *Raoultella* strains.

1-2-5-6 Bacteriocin production :

Bacteriocins were first discovered by Gratia (1925). Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains.

They differ from traditional antibiotics in having a relatively narrow spectrum of action and effect on closely related bacteria (Riely *et al.,* 2001). There are several large categories of bacteriocins which are only phenologically related.

These include the bacteriocin from gram-positive bacteria, the colicins, the microcins, and the bacteriocins from Archeae (Gratia,1925).

The bacteriocin family includes a diversity of proteins in terms of size, microbial targets, mode of actions, and immune mechanisms. The most extensively studied the colcins produced by *E. coli* (Braun *et al.,* 1994; Gouaux, 1997). Colcins gene clusters are encoded on plasmids and are composed of a colcin gene, which encodes the toxin, an immunity gene, which encodes a protein conferring specific immunity to the producer cell by binding to and activating the toxin protein; and a lysis gene, which encodes a protein involved in colcin release through a lysis of producer cell (Riely *et al.,* 2001).

The killing functions range from pore formation in the all membrane to nuclease activity against DNA, rRNA, and tRNA targets (Braun *et al.,* 1994; Gouaux, 1997).

1-2-5-7 Bacteriocin in *Raoultella* spp:

Bacteriocin of *Klebsiella* spp. has been first described by Hammon and Peron (1963), but production of bacteriocin by members of the genus *Klebsiella* was recorded in 1966 (Stouthamer and Tieze, 1966). Few studies reveled that the productions of Klebosien was encoded by gene carried on bacterial chromosome (Sano *et al.*, 1990).

However, the bacteriocins of *Serratia marcesens* are found on both plasmids and chromosomes (Ferrar *et al.*, 1996). In addition to Klebiocins, *Klebsiella* produces a low-molecular weight channel forming bacteriocin called microcin (De Lorenzo and Pugsley,1985).

Price and Sleiqh (1970) attempted to use antibiotics sensitivity patterns and phage and bacteriocin typing to study the epidemiological pattern of outbreak of *Klebsiella* infection in neurosurgical unit. They also found that *Klebsiella* bacteriocins were active also on *Enterobacter* and *Shigella* species and on *E. coli* strains, but they were ineffective on other Enterobacteriaceae (Bauernfeind *et al.,* 1981). The nuclease pyocins of *Pseudomonas auroginosa* are found exclusively on chromosomes (Sano *et al* .,1990).

The range of killing in gram-positive bacteriocins can vary from narrows as in case lactococin to which kills only lactococcus to extradiationaly board as in niacin A, which have been shown to kill a wide range of organisms (Mota-Meria *et al.,* 2000). Whereas, in gram-negative bacteria the killing functions range from pore formation in the cell membrane to nuclease activity against DNA, rRNA, and tRNA targets (Braun *et al.,* 1994; Gouaux, 1997).

1-3 Antibiotic resistance of *Raoultella* spp:

1-3-1 Resistance to β-lactam antibiotics:

Resistance to β-lactam antibiotics could be done due to four mechanisms (Piddock and Wise, 1985; Li *et al.,* 1994; Legakis *et al.,* 1995; Livermore, 1995; Dowson and Coffey, 2000):

a- Hydrolysis of the drug by β- lactamase enzymes.

b- Reduction in the ability of antibiotic to excess its target.

c- Decreased affinity of target penicillin-binding proteins (PBPs).

d- Efflux pumps (pump-mediated resistance).

Hydrolysis of the drug by β-lactamases that breaksdown the structural β-lactam ring of penicillins, is considered as a common mechanism of bacteria causing clinically significant infections (Legakis *et al.,* 1995). In gram-negative organisms, these enzymes are located in periplasmic space to protect penicillin-binding protein from β-lactam attack (Livermore, 1995).

β-lactamases of *Klebsiella* are constitutive and usually are produced at low levels, which nevertheless, are sufficient to protect against ampicillin, amoxicillin, carbencillin, and ticarcillin (Livermore, 1995). Several authors referred to further resistance in *K. oxytoca* via mutational hyperproduction of chromosomal *Klebsiella* enzyme. Hyperproducers have a very characterized antibiogram to penicillin, resistant to cefuroxime

and azteronam, and moderately resistant to cefotaxime and ceftriaxone, but susceptible to ceftazidime (Wu *et al.,* 1991; Liu *et al.,* 1992).

Podschun *et al.,* (1992) showed that antibiotic susceptibility test of strains of *Raoultella* were shown to be comparable in sensitivity to *Klebsiella pneumoniae*. In another study Stock and Widemann (2001) showed that all *Klebsiella* species were resistant or intermediate to amoxicillin, triacillin and to antibiotics to which other Enterobacteriaceae are also intrinsically resistant. *Klebsiella* spp. were sensitive or intermediate to several penicillins, cephalosporins, and susceptible to cefazoline, cephoprazone. They also showed that similar natural susceptibility patterns of *K. planticola* and *K. ornithinolytica* to all tested antibiotics support the status of *K. ornithinolytica* as a biovariant of *K. planticola.* It was found that *K. planticola* strains were resistant to narrow and extended spectrum cephalosporins (Liu *et al.,* 1997; Perilli *et al.,* 1997). In another study, the antimicrobial susceptibility testing of *R. planticola* revealed that all strains tested were susceptible to amoxicillin and calvulnic acid, cefroxime, and tobromycin, and resistant to ampcillin (Podschun *et al.,* 1998).

Recent studies showed that *R. planticola* and *R. ornithinolyitica* are resistant to amoxicillin and triacillin but susceptible to other β-lactam molecules tested and also to

amoxicillin and triacillin were combined with 2μg of calvulante/ml (Walckenaer *et al.,* 2004).

Enterobacterial strains of *Raoultella* species display a pencillinase-related-β-lactam resistance pattern suggesting the presence of a chromosomal *bla* genes. Each gene encoded an ambler class A-β-lactamases, named PLA-1 and ORN-1 for *R. planticola* and *R. ornithinolytica*, respectively (Walckener, *et al.,* 2004). In fact, PLA-1 had stronger hydrolytic activity than TEM-1 not only against cephalosporines, including the extended – spectrum cephalosporins, and aztreonam (Perilli *et al.,* 1997).

1-3-2 Resistance to another antibiotics:

Liu *et al.* (1997) found that *K. planticola* isolates were resistant or reduce susceptibility to azteronam and resistant to nitrofurantoin and gentamicin.

Podschun *et al.,* (1998) found that all *K. planticola* strains susceptible to piperacillin, imipenem, and ciprofloxacin. In another study it was found that all *Klebsialla* species were sensitive to aminoglycosides, quniolones, tetracycline, and trimethoprim and chloramphenicol and nitrofurantoin (Stock and Widemann, 2001).

In one study, all strains of *R. planticola* have a highest level of resistance to gentamicin and ofloxacine (Hostacka, 2001).

In recent study, in France, Brisse and Duijkeren, (2005) showed that *Raoultella* isolates were resistant against ampicillin (99%) and cephalexin (43%), but not against ceftazidime, tetracycline, enorfloxacin, gentamycin and Trimethoprime-sulphamethoxazole.

Stock and Widemann (2001) found that *K. pneumoniae,* subsp. *rhinoscleromitis* strains were more susceptible to antibiotics than strains of *Klebsiella oxytoca*, to antifolate and intermediate to clarithromycin, *K.pneumoniae* subsp. *ozaena*e susceptible to glycopeptides. *Klebsiella* strains were mainly resistant to aminoglycosides (Noriega *et al.,* 1975; Curie *et al.*,1978).

Materials and Methods

2-1: Materials:

2-1-1: Instruments and equipments:

Equipment	Company/Country
Centrifuge	Memmert/Germany
Autoclave	Stermite/Japan
Water bath	Memmret
Micropipette	GT./Britain
Refrigerator	Ishtar/Iraq
Incubator	Memmert
Electric oven	Memmert
Spectrophotometer	Memmert
Compound Light microscope	Olympus/Japan
Sensitive electronic balance	A & D Company/Japan
Shaker water bath	Memmert
Vortex mixer	Memmert
Millipore filter No. 0.22 μm	Sartorius/USA
pH meter	Hoeleze &Cheluis, Germany

2-1-2: Chemicals:

Table (2-2): Chemicals used in the present study

Chemical	Company/Country
Agar-Agar	Sigma/USA
Amyl alcohol	Sigma
Beef extract	Sigma
Brium chloride dihyrate ($BaCl_2$. $2H_2O$)	Fluka/Switzerland
Bromocresol purpule	BDH/England
Calcium carbonate ($CaCo_3$)	Sigma
Calcium Chloride ($CaCl_2$)	Sigma
Cellobiose	BDH
Citric acid	BDH
D-Mannitol	BDH
Dextrose	BDH
2-2 dipyrdil	BDH
EDTA tube	Sigma
Ethanol	Fluka
Galactose	BDH
Fructose	BDH
Gelatin	Sigma

Table (2-2): Contained

Chemical	Company/Country
Glycerol	BDH
Glucoseamine	BDH
Hydrochloric acid (HCL)	BDH
Sulfuric acid (H_2SO)	BDH
Hydrogen peroxide (H_2O_2)	Fluka
Indian ink	China
Potassium di-hydrogen phosphate (KH_2PO_4)	Fluka
di-Potassium hydrogen phosphate (K_2HPO_4)	Fluka
Potassium Hydroxide (KOH)	BDH
L-arabinose	BDH
Lactose	BDH
L-Histidine	BBL/USA
Mannose	BDH
Maltose	BDH
Magnsium chloride	Sigma
Methyl-red	BDH
Di-sodium- hydrogen phosphate (Na_2HPO_4)	Fluka

Table (2-2) : Contained

Chemical	Company/Country
Sodium chloride (NaCl)	BDH
Sodium hydroxide (NaOH)	BDH
α-nephthol	
Amonium chloride (NH4Cl)	Fluka
Ornithine	Fluka
Peptone	BBL
Phenol-red	Fluka
Safranine	Sigma
Sucrose	BDH
Sodium carbonate	BDH
Sodium potassium tartrate	BBL
Sorbose	BDH
Tannic acid	BDH
Tetramethyel-p-pheneyl diamine -dihydrochloride	Sigma
Trichloro acetic acid	BDH
Tri-sodium citrate	BBL
Tryptone	BBL
Urea solution	Fluka
Yeast extract	Sigma

2-1-3 Culture media:

Culture media	Company/Country
Brain-heart infusion agar	Oxiod / U.K
Brain-heart infusion broth	Oxiod
CFA agar	Difco/ USA
Eosin methylene blue agar	Difco
Kligar Iron agar	Difco
MacConkey agar	Difco
MR-VP broth	Biolife-Italy
Muller Hinton agar	Biolife
Muller Hinton broth	Biolife
Nutrient agar	Oxiod
Nutrient broth	Difco
Peptone water	Oxiod
Simmons citrate agar	Oxiod

2-1-4 Antibiotics:

2-1-4-1 Antibiotic disks (Bioanalyse, Turkey)

The antibiotic disks used in the study are listed in Table (2-4).

Table (2-4) : Antibiotic disks used in the study.

Class	subclass	Agent used	Abbreviation	Content
Pencillins	Penicillin	Penicillin	P	10 IU
	Aminopenicillin	Ampicillin	AMP	10 µg
		Amoxycillin	AMX	25 µg
Cephalosporins	1 st Generation cephalosporins	Cephalothin	KF	30 µg
	3 rd Generation cephalosporins	Cephotaxime	CTX	30 µg
Carbpenenmes		Meropeneme	MEM	10µg
Aminoglycosides		Gentamicin	GN	10µg
		Streptomycin	S	10 µg
Ansamycins		Rifampin	RD	30 µg
Tetracyclines		Tetracycline	TE	30 µg
Nitrofurans		Nitrofurantoin	F	300 µg
Quninlones		Nalidixic acid	NA	30 µg
Phenicols		Chloramphenicol	C	30 µg
Fluoroquinolones		Ciprofloxacin	CIP	5µg

2-2 Methods

2-2-1 Solutions and buffers

2-2-1-1 Normal saline solution (0.85%):

This solution was prepared by dissolving 0.85 gm NaCl in 90 ml distilled water (D.W), and further completed to 100 ml with D.W. (MacFaddin, 2000).

2-2-1-2 Phosphate buffer saline:

It was prepared by dissolving 8 gm of NaCl, 0.34 gm (KH_2PO_4) and 1.12 gm (K_2HPO_4) in one liter D.W., the pH was adjusted to 7.3, autoclaved and stored until use (Collee *et al.,* 1996).

2-2-1-3 McFarland tube standard (0.5):

A barium sulfate turbidity standard solution equivalent to a 0.5 McFarland standard was prepared as described by NCCLS (2003a), as follows:

- A volume of 0.5 ml aliquot of 0.048 M $BaCl_2.2H_2O$ (1.175 % w/v) was added to 99.5 ml of 0.18 M H_2SO_4 (1% v/v) with constant stirriting to maintain a suspension.
- Correcting density of the turbidity standard was verified by using reading the absorbance at 625 nm. The absorbance should be 0.08 to 0.10 for the McFarland standard.

- Barium sulfate suspension was distributed in four ml aliquots into screw cap tubes, which were tightly sealed and stored in the dark at room temperature.
- Barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for uniformly turbid appearance.
- Barium sulfate standard should be replaced or their densities verified monthly.

2-2-1-4 Alsever's solution:

It was prepared by dissolving 24.6 gm of glucose, 9.6 gm of tri-sodium citrate, and 5.04 gm NaCl in 1200 ml D.W. The pH was adjusted to 6.1 using 10 % citric acid.All materials were sterilized by filteration in Milipore filter (0.22 μm) (Murray *et al.,* 2003).

2-2-2 Stains and reagents:

The following stains and reagents were prepared as described in MacFaddin (2000):

2-2-2-1 Crystal violet solution:

It was prepared by dissolving 0.5 gm of the crystal violet in 100 ml of D.W. It was used for gram-stain technique.

2-2-2-2 Safranine solution:

It was prepared by dissolving 0.5 gm of the safranine in 10

ml of ethanol (95%) and then completed to 100 ml with D.W. It was used for gram-stain technique.

2-2-2-3 Catalase reagent:

Hydrogen peroxide (3%) was prepared from the stock solution in a dark bottle and used to detection of ability of isolates to produce catalase enzyme.

2-2-2-4 Oxidase reagent:

It was prepared freshly in dark bottle by dissolving 0.1 gm of tetra methyl-p-phenyl diamine dihydrochloride in 10 ml D.W., to detection of ability of isolates to produce oxidase enzyme

2-2-2-5 Kovac's reagent :

It was prepared by dissolving 5 gm of P-dimethyl-amino benzylaldhyde in 75 ml of amyl alcohol. A volume of 25 ml of HCl (0.1N) is added to this mixture. The reagent was placed in dark bottle until use. Using for ability of isolates toproduce indole.

2-2-2-6: Methyl red reagent:

It was prepared by dissolving 0.1 gm of methyl red in 300 ml of ethanol (95%), and then completed to 500 ml with

D.W. for detection complete utilizing of sugars and production acetyl methyl carbinol (butnidol).

2-2-2-7 Vogas-Proskauer reagent:

This reagent consisted of two solutions :

Solution A: α-naphthol : prepared by dissolved 5 gm of : α-naphtol in 100 ml of absolute alcohol (ethanol 96%).

Solution B: 40 % KOH : prepared by dissolving 40 gm of KOH in 100 ml D.W. It was used for detection partial utilizing of sugars and production mixed acid fermentation.

2-3 Preparation of culture and diagnostic media

2-3-1 Ready-prepared media:

Media used in this study (Table 2-3) were prepared in accordance with manufacture's instructions fixed with on their containers. All the above media were sterilized in the autoclave at 121ºC for 15 minutes .After sterilization blood agar base was supplemented with 7.5 % defibrinated human blood, and urea agar base supplemented with 20% sterile urea solution. The media were used for isolation and diagnosis of *Raoultella* species and other bacteria.

2-3-2 Laboratory prepared media:

2-3-2-1 M9 broth: The medium was prepared according to Sambrook and Rusell, (2001) as follows:

Ingredients:

Na_2HPO_4	6 gm
KH_2PO_4	3 gm
NaCl	0.5 gm
NH_4Cl	1 gm

These constitutes were dissolved in 950 ml of D.W, autoclaved, cooled into 50 °C, adding 2 ml from 1 M $MgSO_4$, 10 ml of 20% glucose and 0.1 ml from 1 M $CaCl_2$ (sterilized by filtration) for each one. The volume completed into 1 liter and this medium is used for detection of siderophores and extracellular protease production. M9 medium was used by addition of 2% agar to liquid M9 media , autoclaved and cooled at 50 °C. 0.25 ml of sterilized glucose by filteration with filter 0.22µm and 200 micoromol of 2,2-dipyridil were added for detection presence of siderophore.

2-3-2-2 Motility medium:

This medium was prepared by dissolving the following materials in 1000 ml of D.W.

Tryptone	10 gm
NaCl	2.5 gm
Agar	0.5 gm

and autoclaved. The bacteria were inoculated by stabbing a stright wire carrying the inoculum once vertically into the center of the agar butt to a depth of approximately 2 cm and incubated at 37 ^{o}C for 24 hrs. Formation of cloudy growth out of the line stab indicates the positive result (MacFaddin,2000).

2-3-2-3: Motility ornithine decarboxylase medium:

It was prepared by dissolving the following materials in 1000 ml D.W.

Yeast extract	3 gm
Peptone	10 gm
Tryptone	10 gm
Ornithine	5 gm
Dextrose	10 gm
Agar	2 gm
Bromocresol purpule	0.02 gm

The medium was distributed into tubes and autoclaved (Difco, 1984).

2-3-2-4 Carbohydrate fermentation medium

This medium consisted of :

a- basal medium:

It was prepared by dissolving 10 gm of bacto-peptone, 1 gm of Bacto-beef extract, 5 gm of NaCl, and 0.018 gm of phenol red in 1000 of D.W. The pH was adjusted to 7.4, then the

contents were distributed into test tubes (5 ml portions) and to each tube a durhum tube was located at the bottom. The tubes were then autoclaved.

b- Carbohydrate sources:

The following carbohydrate sources were used; glucose, lactose, sucrose, L-arabinose, cellobiose, maltose, D-manitol , D-mannose, galactose, D-glucoseamine, D-xlyose, fructose, and sorbose. After carbohydrate solution were sterilized by Milipore filter (0.22 μm), they were separately add to the basal medium to give a final concentration 1% of each source. The medium was used to test the ability of bacterial isolates to ferenment a specific carbohydrate incorporated in a basal medium (MacFaddin, 2000).

2-3-2-5 Brain heart infusion glycerol broth medium:

The medium consisted of brain heart infusion as a basal medium, supplemented with 5% glycerol, after autoclaving, it was distributed in 5ml amount, in sterile tubes, then kept at 4°C until use.

This medium was used to preserve the bacterial isolates at -20°C for long term storage (several months) (Collee *et al.*, 1996).

2-3-2-6 Phenol red-tartrate agar medium :

The base of this medium was prepared by dissolving the following materials:

Peptone	10gm
Sodium potassium tartrate	10 gm
NaCl	5 gm
Agar	5 gm
Phenol red	0.024 gm

in 1000 ml of D.W. The pH was adjusted to 6.4 before the addition of indicator . This medium were used to differentiate *Klebsiella oxytoca* form *Raoultella* spp (Difico, 1984).

2-3-2-7 Histamine production medium:

The medium was composed of 0.5% tryptone, 0.5% yeast extract, 2.7% L-Histidine, 0.5% NaCl, 0.1% $CaCO_3$, 2% agar, and 0.006% bromocresol purple, 1000 ml of D.W. The medium was autoclaved for 10 min or less to avoid excessive hydrolysis of agar at the low pH (Niven *et al.,*1981).

2-4 Biochemical tests:

2-4-1 Catalase production test:

Using sterile wood stick, a twenty four hours-old bacterial colony was placed on a clean glass slide and a drop of 3% H_2O_2 solution was added to it. Immediate release of oxygen bubbles indicates a positive result (Baron *et al*.,1999).

2-4-2 Oxidase production test:

Bacterial culture was streaked on nutrient agar plate and incubated at 37 ^oC for 24 hrs. Oxidase reagent was added to the

colonies. Changing the color of the colonies to dark purple indicates a positive result (Baron *et al*.,1999).

2-4-3 H_2S production test:

Kliglar Iron Agar slants were inoculated with bacterial culture by stabbing and streaking, then inoculated at 37^oC for 24 hrs. Formation of black precipitation in the bottom of agar slants indicates a positive test (MacFaddin, 2000).

2-4-4 Carbohydrate fermentation test:

Tubes of carbohydrate fermentation medium were inoculated and incubated at 37 ^oC for 36 hr. Changing the color of the indicator to yellow with or without gas production indicates a positive reaction (MacFaddin, 2000).

2-4-5 Indole production test:

Peptone water broth tubes was inoculated with bacterial culture and incubated at 37 ^oC for 24-48 hrs. Few drops of Kovac's reagent were added to each tube. Formation of pink ring indicates a positive test (Baron *et al*.,1999).

2-4-6 Methyl red test:

Methyl red-vogas-proskaur broth was inoculated with a young culture and incubated at 37 ^{o}C for 24 hours. Few drops of methyl red solution were added to each tube. Changing the color to red indicated complete hydrolysis of sugar and production of acid leading to positive results (Baron *et al*.,1999).

2-4-7 Voges-Proskauer test:

Methyl red-Vogas proskaur broth was inoculated with a young culture and incubate at 37 ^{o}C for 48 hours. About 0.2 ml of 40% KOH and 0.6 ml of α-naphtol solution (2-1-4-7) was added to each tube. After 15 minute, the positive results is represented by the color change to pink red which indicates partial hydrolysis of sugar and production of acetyl methyl carbinol (MacFaddin, 2000).

2-4-8 Citrate (Simmon's) utilization test:

Simmon's Citrate slant was inoculated and incubated with a young culture at 37 ^{o}C for 48-72 hrs. The color change of medium from green to blue indicates a positive result (MacFaddin, 2000).

2-4-9 Ornithine test :

Motility ornithine decarboxylase medium prepared into tubes of bacto-ornithine agar by stabbing with a straight to the

bottom of the medium. The tubes are then incubated for 18-24 hrs at 35 °C. Motility and ornithine decarboxylase reactions are read. The appearance of pink color and no spread of bacteria about needle stabbing indicates a positive result (Difico, 1984).

2-4-10 Urease (Christensen's) production test:

Urea agar slants were streaked with bacterial culture and incubated at 37 °C. Positive result was read after 24 hrs, and every day, for 6 days. Changing the color of medium from to purple pink indicates a positive result (MacFaddin, 2000).

2-4-11 Phenol red-tartarte test:

Phenol red- tartrate agar platese inoculated with ayoung agar culuture and incubated at 37 °C. Growth after 24-48 hrs indicated a positive result (Difico, 1984).

2-4-12 Histamine production test:

Duplicate plates of histamine agar medium were streaked with bacterial culture and incubated, one set at 25 °C and the other at 35°C, for 36 to 72 hrs, then examined for purple colonies with purple hallo on the yellow back ground (positive result) (Niven *et al.,* 1981).

2-4-13 Motility test :

Motility medium tubes were inoculated with bacterial were inoculated by stabbing a stright wire carrying the inoculum once vertically into the center of the agar butt to a depth of approximately 2 cm and incubated at 37°C for 24-48 hrs. Formation of cloudy growth out of the line of the stab indicates a positive result (MacFaddin, 2000).

2-5 Sample collection:

A total of 720 clinical samples were collected in the present study during the period from November 2006 to March 2007. Clinical samples were collected from patients admitted to the main three hospitals in Hilla-city/Babylon province, (Teaching Hospital of Hilla, Mergan Hospital, Maternity and Pediatric Hospital). Types and numbers of clinical samples are shown in Table (2-5).

Table (2-5) :Types and numbers of clinical samples collected during the present study

Sample type	No.
Urine	503
Stool	174
Blood	4
Wound	10
Burn	21
Vagina	3
Total	720

2-6 Identification of bacterial isolates:

Bacterial isolates were identified (Figure 2-1) using the traditional morphological and biochemical tests (Colle *et al.,* 1996; Baron *et al*., 1999; MacFaddin, 2000 and Murray *et al*., 2003).

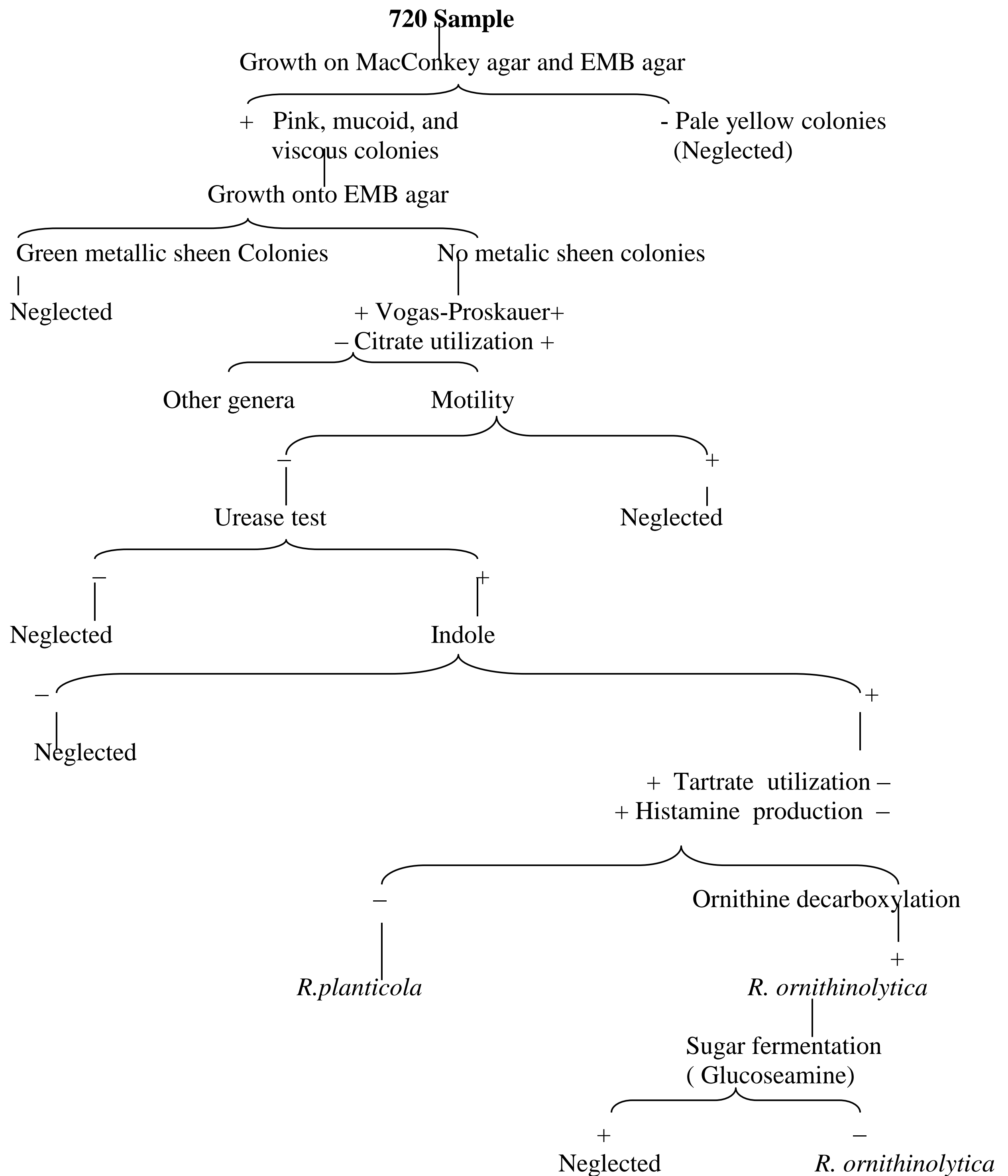


Figure (2-1):Scheme for identification of *Raoultella* spp.(Modefied from MacFddin, 2000 and Drancourt *et al*., 2001, Hansen *et al*., 2004, Alves *et al*., 2006).

2-7 Detection of virulence factors of *Raoultella ornithinolytica:*

2-7-1 Negative staining method (capsule production) :

It is away of using wet amount by putting one drop from Indian ink on surface of a clean slide and using the loop to transport a part of culture (grown on brain heart infusion broth). The part of culture was mixed with Indian ink on slide and covered by cover slip between two filter paper (to prevent aggregation of colony).The slide was examined under the light microscope. Found a clear halo unstained with Indian ink around microorganism indicating the presence of capsule (Murray *et al.,* 2003).

2-7-2 Siderophore production:

The bacterial isolates were examined for growth in the presence of 2,2-dipyridil after incubate at 37°C for 24 hr . Growth in presence 2,2-dipyridil indicates a positive result (Sambrook and Rusell*,* 2001).

2-7-3 Extracellular protease:

Extracellular protease was detected by using M9 media (prepared in 2-1-5-2-3) supplemented with 2% agar (autoclaved 121 °C or 15 minute). After cooling the medium at 50 °C, 0.2% glucose (sterilized by Millipore filter 0.22µm) was added to the medium. The medium was supported by 1% gelatin and

distributed into clean plates. The plates were cultivated by picking and patching and incubated for 24-28 hrs at 37°C. After that for each plate, 3 ml of 5% tri-chloro acetic acid was added (for precipitation of non-lyses protein). The presence of clear zone around each colony indicate the positive result (Priet *et. al,* 1983).

2-7-4 Hemolysin production:

Hemolycin production was showen on blood agar media. The bacterial isolates were obtained after incubation of culture plates for 24°C at 37 °C and detected if any hemolysis presence around the colonies (α or β hemolysis) indicate a positive result (Colle *et al.,* 1996).

2-7-5 Detection of colonization factors antigens:

2-7-5-1 Colonization factor antigen I (CFA/I)

Blood samples of human (type A) and chicken were placed into Alsever's solution heparin tubes and diluted(1:4) with PBS for mannose-sensitive hemagglutination (MSHA) test, with 1 % mannose in PBS for MRHA test. HA tests were performed by the slide agglutination technique as follows; bacterial cells of individual colonies were obtained from cultures grown for 18 hrs on (CFA agar) plates. Growth was picked up with sterile wooden stick and mixed with a drop of the blood (0.02 ml) on a slide at room temperature . The slide was rotated for 2 minutes

and monitored for visible HA, which denoted as MR if the same degree of HA occurred with and without mannose and as MS if HA prevented in the presence of mannose.

2-7-5-2 Colonization factor antigen III (CFA/III) (Tannic acid hemmagglutination):

Blood samples of human (type A) and chicken were placed into Alsever's solution heparin tubes and diluted(1:4) with PBS for tannic acid hemmagglutination. HA tests were performed by the slide agglutination technique as follows; bacterial cells of individual colonies were obtained from cultures grown for 18 hrs on (CFA agar) plates. Growth was picked up with sterile wooden stick and mixed with a drop of the blood (0.02 ml) on a slide at room temperature . The slide was rotated for 2 minutes and monitored for visible HA, in the presence of Tannic acid within 2 minutes at 37 ^{o}C indicates a positive result (Sambrook and Rusell, 2001).

2-7-6 Detection of bacteriocin production:

All *R. ornithinolytica* isolates were examined for their ability to produce bacteriocin by using cup assay method (Al-Qassab and Al-Khafaji,1992) against a sensitive or indicator strain (*E. coli*) (obtained from department of Microbiology/ College of Medicine/Babylon University). The method was as follows:

a- *Raoultella ornithinolytica* isolates were grown into brain heart infusion broth supplemented with 5 % glycerol. All cultures were then incubated at 37 ^{o}C for 18 hrs.

b- Spread the growing bacterial isolates were heavily spread on nutrient agar plates and incubated at 37 ^{o}C for 18 hrs.

c-Agar disks were cut from the culture agar layer using sterilized 5 mm cork borer.

d-A volume of 0.1 ml indicator isolates (*E. coli*) grown for 2-3 hrs in shaking water bath at 37 ^{o}C (to obtain 10^6 – 10^7 cells/ml) in nutrient broth, was spreaded on nutrient agar plates.

e- Agar disks were transferred carefully to the agar surface seeded with indicator strain and incubated overnight at 37°C.

f- Sensitivity patterns were recorded, presence of inhibition zone around the agar disks indicated a positive result.

2-8 Collection of serum sample:

blood samples (3-5) ml were drawn from aseptically in 5 ml disposable syringe. Blood sample left to clot at room temperature for 30 minute. After that clot was detached by sterile disposable inoculating loops. The tubes were centrifuge at 3000 rpm for10 minutes. Cells free-sera were collected in sterile test tube , numerated at (-20 ^{o}C) (Lewis *et al*., 2001).

2-8-1 Serum bactericidal assay:

Bacterial isolates (*E. coli, Enterbacter* spp., (obtained from department of Microbiology/College of Medicine/Babylon

University) and *R. ornithinolytica*) were grown in nutrient broth for 4-6 hrs at 37 °C, harvested, and adjusted to a concentration of 2 x 10^6 bacterial cells/ml with physiological saline. Bacterial suspension 25 µl was mixed with 75µl of pooled normal human serum in sterilized test tubes. Viable counts of bacteria were determined according to MacFadin *et al*., (2000) after incubation for 1, 2, and 3hrs. Each isolate was tested at least three times. Isolates were considered serum resistant or serum sensitive if the grading was the same in all experiments. Mixture of bacterial suspension and normal saline was used as control (Sahly *et al*., 2004).

2-9 Antibiotic disk susceptibility test:

The antimicrobial susceptibility of the bacterial isolates to antibiotics was determined using disk diffusion method (NCCLS, 2003b). The antibiotics used are listed in Table (2-4). Tests were performed on plates of Muller-Hinton agar as follows:

- A portion of 0.1 ml of bacterial suspension equal to A 0.5 McFraland suspension was spreaded on plates, then dried in an incubator at 35 °C for 15 minutes.
- Antibiotic disks were placed on the agar with a sterile forceps (6 disks for each plate). The agar plates were incubated at 35°C for 16-20 hours.

- Results were recorded by measuring the inhibition zone (in millimeters) and interpreted according to the National Committee of Clinical Laboratory Standards documents (NCCLS, 2003b).

2-10 Preservation and maintenance of bacterial isolates

The bacterial isolates were preserved on nutrient agar slants
(table 2-3) at 4°C. The isolates were maintained monthly during the study by subculturing on new culture media. For long preservation, brain heart infusion broth supplemented with 15% glycerol was used, and the isolates were maintained frozen for 6-8 months at - 20 ^{o}C (Collee *et al.*, 1996).

2-11 Statistical analysis: The χ^2 (Chi-square) test was used for statistical analysis. $P<0.01$ was considered to be statistically significant.

3-1 Isolation and identification of bacterial isolates:

In this study, 720 clinical sample were collected from three main hospitals in Hilla city–Babylon Province, during the period from November 2006 to March 2007. Clinical samples were collected from 400 males and 320 females patients. A total of 144 clinical isolates were identified as *Klebsiella*-like organisms, out of these, 11 isolates *Raoultella* spp. were identified which represent (7.6%) of all *Klebsiella*-like organisms .

The goal of the present study was to identify *Raoultella* species and to ascertain the detection rate of *Raoultella* from the other *Klebsiella* species as well as the bacterial pathogens presented in clinical samples. Some biochemical characteristic of *Raoultella* have been exploited in the isolation and identification of this genera. An important characterization is that , *R. ornithinolytica* exhibited positive reactions for indole test and able to decarboxlyase of ornithine (Drancourt *et al.,* 2001; Hansen *et al*.,2004).

The result of microscopic examination showed that *R. ornithinolytica* isolates are gram negative, non spore forming, bacilli. Result of biochemical tests (Table 3-1) showed that all the isolates of *Raoultella* were fermented glucose, lactose, sucrose, L-arabinose, D-mannose, D-mannitol, maltose, D-xylose, and unable to ferment D-glucoseamine. The isolates exhibited positive reaction for indole, citrate, urease,

voges-proskaur, and ornithine decarboxylase,and negative reaction for histamine production, tartarate utilization .

Table (3-1): Morphological and biochemical tests of 11 *Raoultella ornithinolytica* isolates

Test	*R. ornithinolytica*
Gram stain	-*
Catalase	+**
Oxidase	-
Growth at 10 ºC	+
H_2S production	-
Acid from:	
Glucose	+
Lactose	+
Sucrose	+
Sorbose	+
L-arabinose	+
Cellobiose	+
D-mannose	+
D-manitol	+
Maltose	+
D-xylose	+
D-glucoseamine	-
IMViC test:	
Indole	+
Metyl Red	+
Vogas-Proskauer	+
Citrate utilization	+
Urease	+
Ornithine decarboxylase	+
Motility	-
Histamine production	-
Kligar Iron Agar	Acid/ Acid
Tartrate utilization	-

* - NEGATIVE

** + POSITIVE .

According to biochemical test all 11 isolates of *Raoultella* were diagnosed as *R.ornithinolytica,* whereas, *R. planticola* and *R. terrigena* were not isolated in this study. All results of tests in the Table (3-1) agreed with tests that mentioned by Sakazaki *etal.,*(1989), Drancourt *et al.,* (2001) and Hansen *et al.,* (2004); Alves *et al.,*(2006).

The present study represented first to investigate the occurrence of *Raoultella* species isolates in human clinical samples and represent the first record of the occurrence of the *R. ornithinolytica* in human clinical specimens in Iraq.

This result agreed with other studies which found that the percentage of *Raoultella* recovered from clinical specimens were ranged between 8-9% (Mori *et al*., 1989; Monnet *et al.,* 1991; Podschun *et al*., 1992; Podschun *et al*., 1998; Al-Chracckh, 2007). Mori and his colleagues (1989) found that *R. planticola* represented 8% of all clinical *Klebsiella* spp. isolated from Nagoya University Hospital and several regional general Hospital in Japan. Moreover, *R. planticola* represented 8% from 204 *Klebsiella* isolates in France (Monnet *et al.,* 1991). Podschun and his colleagues (1992) reported that *R.planticola* isolated from neonates in intensive care unit (ICU) represented 8.7% of 131 *Klebsiella* isolates in Germany. In another research, they reported that the isolation of *R. planticola* from neonates in neonatal ward represented (9%) of all *Klebsiella* spp found in Germany (Podschun *et al.,* 1998).

Also the results in the present study were approximately compatible with results obtained by Al-Charrakh (2007) who found that *R. planticola* represented (6%) of all clinical *Klebsiella* isolates recovered in Hilla-city. However, in another studies *R. planticola* represented only (1%) of clinical isolates in Georgia and Brazil, respectively (Westebrook *et al.,* 2000; Alves *et al.,* 2006). *R.planticola* isolates were not isolated in the present study, and this result agreed with the finding obtained by Watanakunakron and Jura (1991) who showed that *R. planticola* isolates were not appeared in any case in the area of study in United States. The third species of *Raoultella,* is *R. terrigena* also was not recovered in this study, and this result agreed with finding obtained by other investigators who reported that *R.terrigena* isolates were not found or may be found in low percentages in human clinical samples compared with *R. planticola* (Podschun *et al.,* 1992 ;Westebrook *et al*., 2000 ; Alves *et al.,* 2006).

Podschun *et al.* (1992) found, in a three years-survey conducted from 1988 to 1990 on *Klebsiella* species isolated from human clinical sample, that only ten strains of *Klebsiella terrigena* (0.4 %) were isolated among 2355 indole-negative *Klebsiella* isolates.

The result of the present work agreed with Westebrook *et al.* (2000) who found that geographical differences may affect

in the present study, found that most isolates 8 (72.7%) of the 11 *R. ornithinolytica* were isolated from 174 rectal swab or stool samples (Table 2-5) and this result agreed with the finding obtained by Kuhn *et al.,* (1993) who found that the principle reservoirs of transmission of *Klebseilla* in hospital setting are the gastrointestinal tract of patient and hand of hospital personal. Moreover, this result in the present study agreed with Podschun *et al.,* (1998) who showed that a high rate (68%) of *Klebsiella* was isolated from feaces.

The result also agreed with the finding obtained by Selden *et al.,* (1971) who found that intestinal colonization considered as the main reservoir for *R. planticola*. The result was also confirmed by the finding obtained by Podschun *et al.,*(1998) who found that in most children, *Raoultella* could be detected in rectal swab only. In the present study, only 3 (27.3%) isolates of *R. ornithinolytica* were detected in 503 urine samples, a lonely, and were not detected in blood, vagina, ear, and wound samples.

The absence of *R. ornithinolytica* in these clinical samples attributed to the limited number of samples taken in this study .

The specific and non-specific local defenses of these environments may be prevent the growth of these bacteria. The percentage (7.6%) of *R. ornithinolytica* isolates observed in this study indicates the presence of a cryptic bacteria at a

surprisingly high frequency in human clinical samples that are not detected by previous studies in Iraq.

The present study showed that 7 (63.6%) isolates of the 11 *R. ornithinolytica* were isolated from children, This result agreed with finding obtained by Podschun *et al.,* (1998), who found that *R. planticola* isolates were detected in most children. Two (18.2%) isolates of *R. ornithinolytica* were isolated from infant and this result agreed with finding obtained by other studies which recorded that *Klebsiella* infection are remarkably troublesome, particularly in premature infants and intensive care unit, pediatric patients are easily colonized by *Klebsiella* spp (Hart, 1993; Kuhn *et al.,* 1993). Also, results revealed that 2 (18.2%) isolates of *R. ornithinolytica* were isolated from adults urine sample. This result confirmed by the finding obtained by Hart, (1993) who found that immunocomprised patients especially elderly people easily are colonized by *Klebseilla* spp. and agreed with the finding obtained by Westebrook *et al.,* (2000) who found that *Raoultella* spp. are responsible for nosocomial infection in adult in Georgia hospital.

Monnet and Freney (1994) showed that the clinical significance of *R.ornithinolytica* remains unknown. The clinical significance of *R. ornithinolytica* remains obscure, even thought clinical isolates of this species are not uncommon (Stock and Widemann, 2001).

3-2 Virulence factors of *Raoultella ornithinolytica*:

Virulence factors of *R. ornithinolytica* demonstrated in this work were included production of capsule, siderophore, bacteriocin, colonization factor antigens (CFA/I, and CFA/III), and serum resistance.

3-2-1 Capsule production:

Results in the present study found that all *R. ornithinolytica* isolates were able to produce capsule (Table3-2). Capsule production is considered as an important virulence factor in bacterial pathogencity and protect the bacterium from phagocytosis by polymorphnuclear leukocytes and prevent killing by bactericidal serum factors (William's *et al.,* 1983; Simmon- Smith, 1986; Podschun *et al.,* 1992).

3-2-2 Hemolysin and siderophore production:

The results of this study revealed that all isolates of *R. orinthinolytica* were unable to produce hemolysin on blood agar, and 9 (81.8%) of 11 isolates were able to produce siderophore (in presence of dipyridil).These results are similar to that reported by Pyanne, (1988), Trakkanen *et al.,* 1992; Podschun *et al.,* (1993), who found that *Klebsiella* spp. have the ability to provide iron and produce siderophore without hemolysin production. Production of siderophore by *Klebsiella*, was also confirmed by Nassif and Sansonetti (1986) who found

that *Klebsiella* isolates have an ability to produce siderophore in presence of dipyridil. According to the result of the present study, there is an association may be present between aerobactin synthesis and the virulence factor of *R. orinthinolytica* isolates.

3-2-3 Colonization factor antigens:

The results of this study revealed that all isolates of. *R. ornithinolytica* that have been isolated from urine and stool samples have able to express the colonization factor antigens (CFA/I, and CFA/III) (Table 3-2).

The expression of colonization factors antigens (CFA/I, and CFA/III) by *R. ornithinolytica* isolates may play an important role in pathogenesis and virulence of these bacteria which enhance their ability to colonize the intestinal and urogenital tracts. This result agreed with findings obtained by other studies which showed that *Klebsiella*, *Raoultella* expressed type (I, III) pili which mediate bacterial binding to mucous or epithelial cell of urogenital, respiratory and intestinal tract (Ofek and Beachey, 1978; Fader and Davis,1980; Blaish *et al.,*1982; Trakkanen *et al.,* 1990; Trakkan *et al.,* 1992; Podschun *et al.,* 1993; Venegas *et al.,* 1995; Podschun *et al.,* 2000).

The present study showed that all *R. ornithinolytica* isolates were produced CFA/I (Table3-2) expressed by hemagglutination of human red blood cells (group A) in

presence of mannose and this result agreed with findings obtained by Evans *et al.,* (1977); Evans and Evans,(1978) who found that CFA/I cause mannose-resistant hemagglutination which agglutinate human group A erythrocytes, and agreed with finding obtained by Maayan *et al.* (1985), who found that *Klebsiella pneumoniae* that have CFA/I classified under " mannose specific" bacterial lectins .

Erythrocytes receptors responsible for attachment by E.coli possing CFA/I is a sialoglycoconjguate (sialoglycoprotin) isolated from erythrocytes membrane. Glycoconjguates containing sialic acid inhibited the hemagglutination reaction (Bartus *et al.,* 1985). CFA/I is a carbohydrate biding fimbriae which specifically intracts with a number of carbohydrate sequences that present in human intestine and erythrocytes group A glycosphingolipid fraction. CFA/I bound in the mono-di-tri glycoslycremide region in the non-acid glycosphingolipid fraction. The non-binding of CFA/I to sialic acid containing glycosphingolipid were not recognized and the binding for pure glycosphingolipid (Jannson *et al.,* 2006).

Table (3-2): Virulence factors of *Raoultella ornithinolytica* isolates

Isolate designations	Clinical sample	Capsule	Siderophore	Hemolysin	Colonization factor antigen		Extracellular protease	Bacteriocin Dimeter in mm
					CFA/I	CFA/III		
*R.ornithinolytica*398	urine	+	+	-	+	+	-	+(**34**)
*R.ornithinolytica*487	urine	+	+	-	+	+	-	-
*R.ornithinolytica*104	urine	+	+	-	+	+	-	+(**12**)
*R.ornithinolytica*26	stool	+	+	-	+	+	-	+(**34**)
*R.ornithinolytica*13	stool	+	+	-	+	+	-	-
*R.ornithinolytica*27	stool	+	+	-	+	+	-	-
*R.ornithinolytica*32	stool	+	+	-	+	+	-	+(**12**)
*R.ornithinolytica*70	stool	+	-	-	+	+	-	-
*R.ornithinolytica*99	stool	+	+	-	+	+	-	+
*R.ornithinolytica*163	stool	+	-	-	+	+	-	-
*R.ornithinolytica*45	stool	+	+	-	+	+	-	-

The present study showed that all *R. ornithinolytica* isolates produced CFA/III (Table3-2) by hemagglutination of red blood cell group A in presence of tannic acid. This result agreed with finding obtained by Trakkanen *et al.* (1990) who found that *Klebsiella* mediates mannose-resistant hemagglutination tannin trated erythrocytes.

These results are approximately similar to the findings obtained by Podschun and Sahly, (1991) and Podschun *et al.* (2000) who found that (69-89%) of all *Klebsiella* isolates from urinary and intestinal tract infections were able to express CFA/III.

CFA/III with strongly hemagglutination, suggested that the appendage do function in vitro as adhesion (Gerlach *et al.,* 1989). *Klebsiella pneumoniae* have a fimbial adhesion (MrkD) {mannose resistant *Klebsiella* (D) poly-peptide} of CFA /III that composed of amino acids that are necessary for substrate which binding specificity are located throughout the length of MrkD polypeptide. Hydrophilic amino acid is found to be necessary for hemagglutination reaction (Hornick *et al.,* 1995).

3-2-4 Extracelluler protease:

The results expressed in Table 3-2 showed that all isolates of *R. ornithinolytica* were unable to produce protease and these results fitted with fact that *Klebsiella* were unable to produce extracellular protease (gelatinase). The extracellular enzymes of

bacteria like protease, lipases, and nuclease are not shown to have a direct role in invasion or pathogenesis, but these enzymes presumably may associate in bacterial nutrition or metabolism (Brooks *et al*., 2004) .

3-2-5 Bacteriocin production of *Raoultella ornithinolytica*:

The ability of *R. ornithinolytica* isolates to produce bacteriocin was tested. Localy clinical isolates of *R. ornithinolytica* were used as bacteriocin-producing isolates and an *E. coli* isolate was used as a sensitive (Indicator) strain. Results of the present study showed that 4 (36.4%) of *R. ornithinolytica* isolates were able to produce bacteriocin (Table 3-3). These include *R. ornithinolytica* 398, *R. ornithinolytica*487, *R. ornithinolytica*104; and *R. ornithinolytica* 26 isolates *R. ornithinolytica*398 ,and 26 were expressed clear and relatively large inhibition zones (34 mm) (Table 3-3). The production of bacteriocin by *Klebsiella* and *Raoultella planticola* strains was recorded by several authors (Bauerenfeind *et al.,*1981; Shagra and Turianitsa,1993).

In this study, all bacteriocin producing isolates were isolated from urine and stool samples and this finding assumed the bacteriocin may be a virulence factor of *Raoultella* pathogencity. Morever, It was found that the cytolysin of *Enterococus faecalis* posses both hemolysin and bacteriocin

which may increase the presistance of these bacteria in blood stream and their resistance to serum, indicating that the bacteriocin is essential for virulence and pathogencity of *Enterococcus* in septicemia (Ike *et al.,* 1984; Coburn, *et al.,*1997).

On other hand, Viddotto *et al.* (1991) found that the bacteriocin activity of *E. coli* isolates is not essential for virulence and pathogencity of producing isolates but it aids them in their competition.

3-3 Antibiotic resistance of *Raoultella ornithinolytica*:

The antibiotic resistance of all the 11 *R. ornithinolytica* obtained in the present study was determined (Figure 3-1). All these isolates were found to be (100%) resistant to penicillin, ampicillin , gentamycin, chloromphincol, rifampin, cephalothin, cefotaxime, lincomycin, streptomycin, amoxicillin, calthromycin, azithromycin, and (90.9%) resistant to tetracycline, (63.6%)were resistant to naldixic acid (Figure 3-1). This result agreed with other studies (Liu *et al.,* 1997; Stock and Widemann, 2001; Brisse and Duijkeren, 2005), which found that all strains of *Klebsiella* spp are resistant to ampicillin, pencillin and amoxicillin. The Results also showed that all isolates of *Raoultella ornithinolytica* (100%) were resistant to cefotaxime and cephalothin (Figure 3-1).The result in the present study is compatible with other researchers who found that

chromosomally encoded resistance to first and second generation cephalosporins in strains of *Klebsiella* has been emerged in many hospitals (Sykes and Matthow, 1976; Liu *et al.,* 1997 and Priol *et al.,* 2000).

Result in the present study also showed that all *Raoultella ornithinolytica* were resistant to gentamycin and streptomycin (Figure 3-1), and this result agreed with findings obtained by several studies which showed that strains of *Klebsiella* have a highest level of resistant to gentamycin and other aminoglycoside antibiotics (Noriega *et al.,* 1975; Hostocka *et al.,*2001).

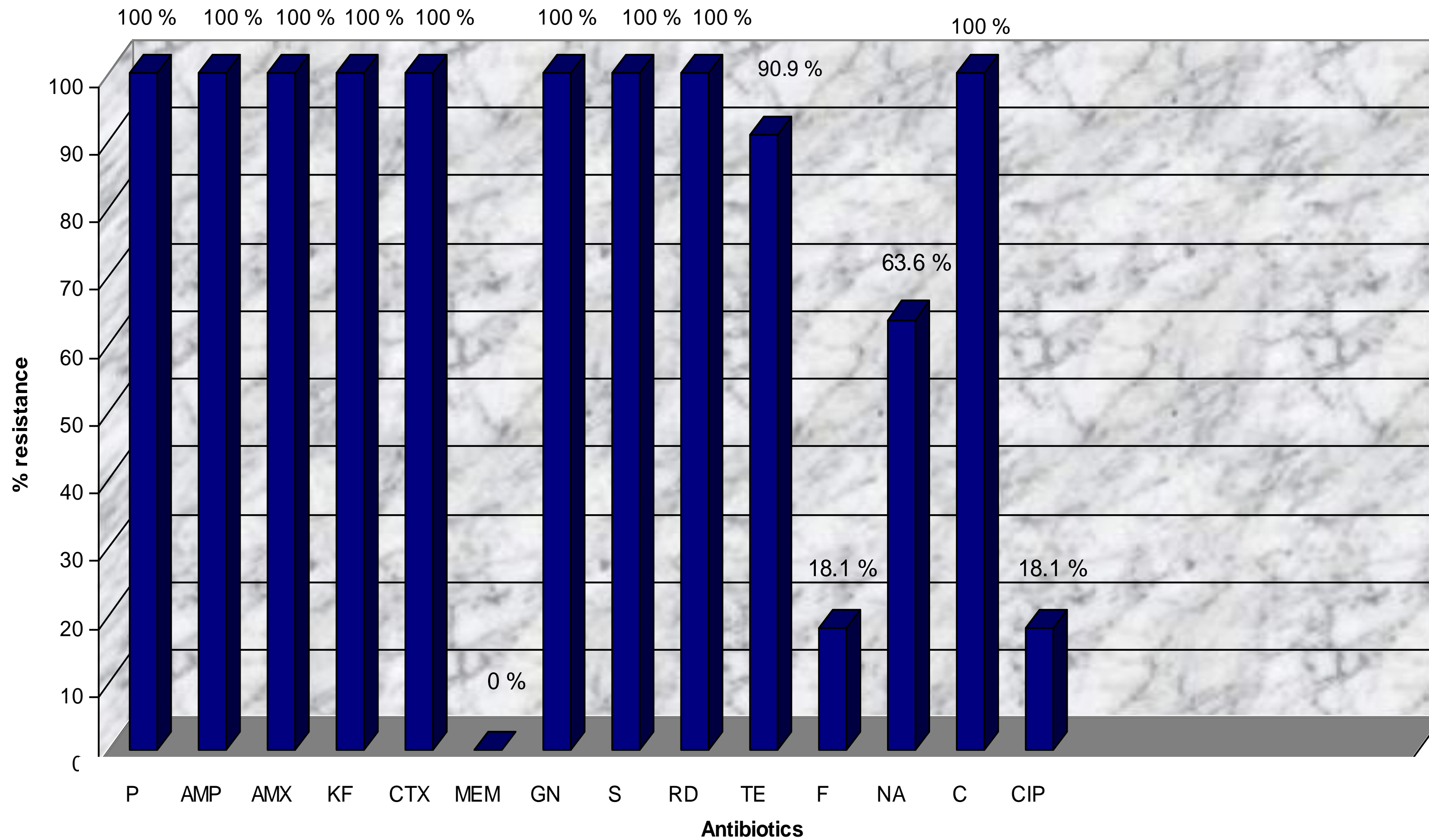


Figure (3-1): Resistance of 11 *R. ornithinolytica* isolates to 14 antibiotics.

P: Penicillin, AMP: Ampicillin, AMX: Amoxycillin, KF: Cephalothin, CTX: Cephotaxime, MEM: Meropeneme, GN: Gentamicin, S: Streptomycin, RD: Rifampin, TE:Tetracycline, F:Nitrofurantoin, NA:Naldixic acid, C:Choroampnicol,,CIP:Ciprofloxacine

The results also showed that (90.9%) of *Raoultella ornithinolytica* isolates were resistant to tetracycline and (63.6%)of them were resistant to naldixic acid (Figure 3-1).

These results were similar to results obtained by Bauernifined *et al.,* (1993) who found that *K. pneumoniae* is resistant to tetracycline .

By contrast, other study showed that *Klebsiella* spp were sensitive or intermediate to several pencillins, all cephalosporins, aminoglycosides, quinolones, tetracyclins, chloromphincol, and nitrofurantoin (Stock and Widemann, 2001). Results in Figure (3-1) also showed that all isolates of *Raoultella ornithinolytica* were sensitive to meropenem and (18.1%) of them were resistant to both ciprofloxacin and nitrofurantoin. This result is compatible with finding obtained by other study who found that all strains of *Raoultella* were sensitive to impenem and ciprofloxacin (Podschun *et al.,*1998).

Depending on the results of antibiotics sensitivity observed in this study, *Raoultella ornithinolytica* may be regarded as multi-drug resistant by expressing resistance to more than (14) antibiotics and this result may be compatible with findings obtained by Oplustil, *et al.,* (2001); Winokunet *et al.,* (2001); Al-Charrakh (2005); Al-Charrakh (2007) who showed that *Klebsiella*, *Raoultella planticola*, expressed multi-drug resistance to several antibiotics.

The antibiotic resistance is associated with a high degree of pathogencity and ability to act as a true pathogen that may cause dangerous infections diseases. Since *R. ornithinolytica* isolates were sensitive to meropenem, ciprofloxacin and nitrofurantoin, these drugs may be considered as drugs of choice for the treatment of infections caused by these microorganisms.

3-4 Serum resistance of *Raoultella ornithinolytica* :

Figure (3-2-a) shows the effect of human serum on the growth of *E. coli* , *Enterobactor* spp and *R. ornithinolytica* 398 after one hr. of incubation on nutrient agar . *R. ornthinolytica* was the most sensitive to the effect of serum, when compared to the other bacterial types, on the same manner, *E. coli* was less effected by serum .

On other hand the result expressed in Figure (3-2-b) showed the high effect of serum on the growth of all bacterial isolates after 2 hr, of incubation. After 3 hr, of incubation *R. ornithinolytica*, was the most senstive bacterial isolate when compared to the other isolates(Figure 3-2-c).

The pathogencity of bacteria that invade the blood stream is partly a function of their ability to evade the bactericidal effect of serum , which is mediated by the complement cascade (Simberkoff *et al.,* 1976).

Commensal bacteria are generally vulnerable to the bactericidal effect of serum , while nosocomial bacteria tend to

be much more serum resistant (Schoolnik *et al.,* 1976; Simberkoff *et al.,* 1976; Olling, 1977, Abbas and Lishtman, 2007).

The results in Figure (3-2 a, b, c) Statistically by using χ^2 test showed that there was a strong relationship between the bacterial types and serum resistance ($P< 0.01$) . Results also showed that the serum resistance profile of *E. coli* and *Enterobacter* spp. after first hour of incubation tends to be similar to that of after third hour of incubation. After two hours of incubation , both these bacterial isolates were sensitive to the serum by comparing to the relative rate of growth in control (normal saline), this may be due to the fact that serum components may need to time to exhibit their effect on bacterial growth which was clearly observed after 2 hrs., but after 3 hrs. bacterial isolates may develop a mechanism of induced resistance that makes bacterial isolates able to exhibit a degree of serum-tolerance. *R. ornithinolytica* exhibited a high degree of serum sensitivity when compared with the growth rate in control.

The serum-senstive profile of *R.. ornithinolytica* may explain that this bacteria did not isolate from samples, in this study. This senstivey may limit the pathogenic properties of *R. ornithinolytica* in normal subjects (immunocomptence), at the same time the pathogenic capacity increases in immunocompromized patient as well as in age extremes.

Most strain of gram-negative bacteria are sensitive to the bactericidal effect of human serum, whereas, pathogenic strains often exhibit serum resistance properties (Olling, 1977).

The feature "serum resistance" has been correlated with onset of infection and symptoms (Gower *et al.,* 1972).

The main role of the serum bactericidal systems is thought to prevent microorganisms to invading and persisting in the blood. Even differences in the degree of bacterial serum susceptibility may determine whether a strain is able to infect as well as the length of time it takes the organisms to establish the infection (Taylor, 1983).

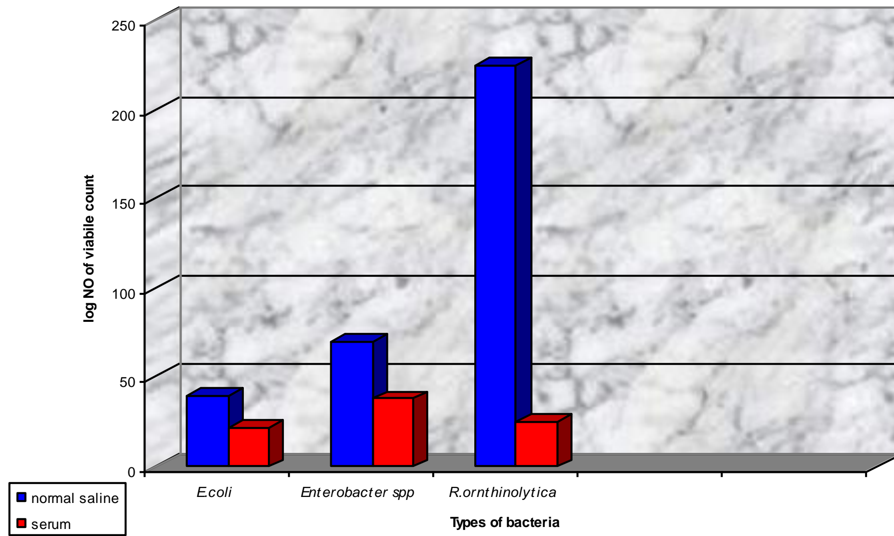
After the onset of inflammation, invading *Klebsiella* strains meet the cellular and humoral bactericidal components of the invading microorganisms includes the primarily by complement proteins (Rich *et al .,* 2003).

Lipopolysaccharides (LPS) have been implicated as a major factor in the ability of bacteria to resist serum bactericidal activity by the host (Tomas *et al.,* 1986; Ciurana and Tomas,1987; Porat *et al.,* 1987; Merino *et al.,* 1992).

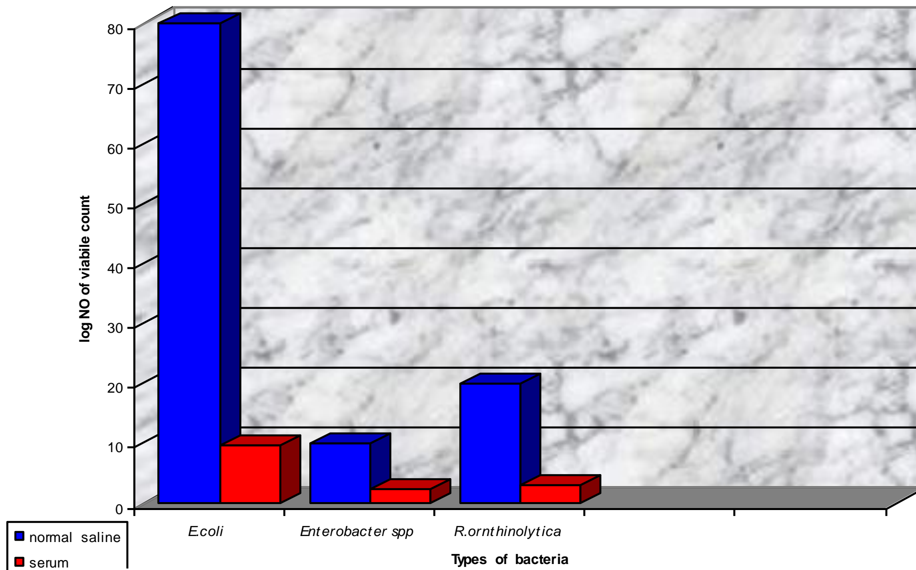
The serum bactericidal activity is mediated primarily by complement proteins. After their cascade-like activation, these proteins accumulate as membrane attack complex (MAC) on the surface of microorganisms (Taylor, 1983).

This complex consists of the terminal complement proteins C5b-C9 which produce a transmembaranace pores in the outer membrane of gram-negative bacteria (Ramm *et al.,* 1983; Rich *et al .,* 2003).These pore cause an influx Na^+ and subsequent osmotic lysis of bacteria.

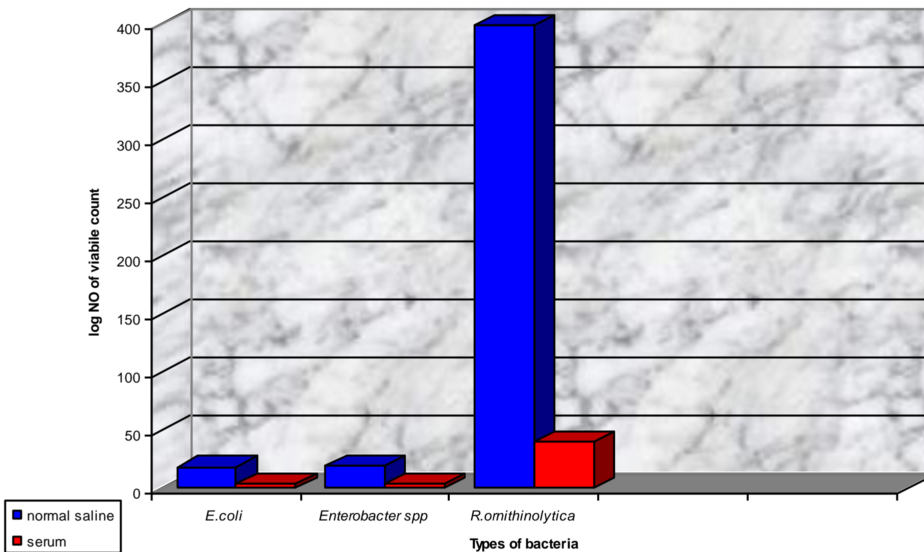
The molecular bases of serum resistance is not entirely clear (Taylor and Kroll, 1985).



a- Anti- serum activity for *E.coli* , *Enterobacter* spp and *R.ornithinolytica* after1 hr of incubation.



b- Anti-serum activity for *E.coli* , *Enterobacter* spp and *R.ornithinolytica* after 2 hour of incubation.



Figure(3-2-c): Anti -serum activity for *Ecoli*, *Enterobacter* spp and *R.ornithinolytica* after 3 hour of incubation.

Figure (3-2 a, b, c): Anti - serum activity for *E. coli*, *Enterobacter* spp, and *R.. ornithinolytica* after 1, 2 and 3 hours of incubation.

The O-side chains of the LPS of pathogenic bacteria are thought to play a major role in conferring serum resistance.

Gram negative bacteria which express intact O-side chain (S-LPS) are generally more serum resistant than those which do not express O-side chains (R-LPS) (Taylor, 1983).

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