Pathogenicity Study of a Number of Gram- Negative Bacilli Isolated from Clinical Samples

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Abstract

Fifty isolates from different clinical samples (urine and wounds) were collected from Al-Nu'man General Hospital in Baghdad city. Sampling activates were carried out from 23April to 23June 2014. The age of patient range between 40- 70 years . The Gram-negative isolates were subjected to antibiotic susceptibility, β-lactamase production (86%), biofilm formation (84%), hemolysin production (48%).The resalt appered that *Escherichia coli* (54%) was the most common followed by *Proteus vulgaris* (18%), *Enterobacter aerogenes* (8%), *Proteus mirabilis* (6%), *Enterobacter cloacae* (4%), *Acinetobacter baumannii* (4%) and (2%) for each *Klebsiella oxytoca*, *Enterobacter gergaviae* and *Pseudomonas mendocina*. Gram negative isolates were observed resistance (100%) for Ampicillin, Carbenicillin, Cefalothine, Cefotaxim and Meropenem. Most isolated bicteria showed less resistance to Amoxicillin / clavulanic acid and Ceftazidime, also they showed sensitivity to Imipenem.

Plasmid profile analysis, *Proteus vulgaris* isolates showed one large plasmid only.

Key words: Gram negative, Multidrug resistance, β-lactamase, biofilm, hemolysin, plasmid.

دراسة امراضية بعض البكتريا العصوية السالبة لصبغة كرام المعزولة من بعض الحالات السريرية

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الملخص

تم جمع 50 عينة من حالات سريرية مختلفة (شملت التهابات المجاري البولية والتهابات الجروح) من مستشفى النعمان العام في بغداد للفترة من 23 نيسان لغاية 23 حزيران 2014 وكانت اعمار المرضى تتراوح بين 40 -70 سنه ؛ وكانت جميع العزلات التي تم الحصول عليها من انواع البكتريا السالبة لصبغة كرام حيث اظهرت(54%) تعود الى بكتريا *Escherichia coli* تليها بكتريا *Proteus vulgaris* (18%) و *Enterobacter aerogenes* (8%) و *Proteus mirabilis* (6%) تليها كل من *Enterobacter cloacae* وبكتريا *Acinetobacter baumannii* وبنسبة (4%) لكل منهما ؛ واظهرت (2%) لكل من *Klebsiella oxytoca*, و *Enterobacter gergaviae* و *Pseudomonas mendocina*. تم الكشف عن انتاج البكتريا لانزيمات البيتالاكتاميز وبلغت (86%) ؛ وانتاج انزيمات الهيمولايسين وبلغت (48%) ؛ وقابلية البكتريا على تكون الغشاء الحيوي وبلغت (84%).

اجري فحص الحساسية للمضادات الحيوية وقد اظهرت جميع العزلات مقاومة (100%) لكل من مضادات Ampicillin , Carbenicillin, Cefalothine, Cefotaxim, Meropenem. .
واظهرت اغلب العزلات مقاومة اقل لكل من مضاد Amoxicillin / clavulanic acid و Ceftazidime وكانت اغلب العزلات حساسة لمضاد Imipenem . واجرى عزل البلازميدات للعزلات البكترية المختلفة وأظهرت النتائج انه عزلة واحده تعود الى بكتريا *Proteus vulgaris* تمتلك بلازميد كبير .
الكلمات الرئيسية: البكتريا السالبة لصبغة كرام ؛ المقاومة المتعدده للمضادات الحيوية ، انزيمات البيتالاكتاميز ، الهيمولايسين ؛ الغشاء الحيوي ، البلازميدات.

Introduction

Gram-negative bacteria cause infections including pneumonia, bloodstream infections, wound or surgical site infections and meningitis (1). The most important Gram-negative bacilli are members of the family Enterobacteriaceae such as *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter* and *Proteus*, (2) normal intestinal flora of humans, animals and may be isolated from a variety of environmental sources. Previous study showed 87.8% from clinical isolates was gram negative bacilli (3). *E.coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Klebsiella spp*., *Haemophilus influenza*, *Proteus spp*. and *Enterobacter spp*. Most common gram negative bacteria isolated from clinical samples (4). Multidrug-resistant Gram-negative organisms (MDRGNs) have emerged as a major threat to hospitalized patients and have been associated with mortality rates ranging from 30 to 70% (5).

Several virulence factors may be responsible for the pathogenicity of the bacteria. Extended spectrum β-lactamases (ESBLs) were found in 63.6 % *E. coli* and 66.7% *K. pneumonia* isolates (6) and also found in *Proteus species* and *Acinetobacter species* (7,8). The bacterial biofilms in the human body is a major cause of recurrent or chronic infections (9). There are four basic criteria to define biofilm-associated infections: (i) Bacterial cell adherence to or association with a surface, (ii) *In vivo* observation of bacterial cell clusters, (iii) a localized infection pattern and (iv) increased resistance to antibiotic treatment in the host compared to resistance of genetically equivalent planktonic bacteria (10). Previous study showed *Escherichia coli* isolates were assessed for their ability to produce biofilm *In vitro* by slime production (11). Hemolysin is a cytolytic protein capable of lysing human, horse and rabbit erythrocytes (1), the haemolysin production analysis showed that 96.6% of *Enterobacteriaceae* produced cytolytic protein toxins (α-haemolysin) (12), and β-hemolysin production by *Proteus spp.*and *E.coli* (13,14).

Plasmid profile analysis is useful in determining the epidemical strain in outbreaks caused by multiple species: *Escherichia*, *Klebsiella*, *Pseudomonas*, *Serratia* and *Streptococcus* (15). Various diverse phenotypic characteristics are encoded by plasmids, these include antibiotic and metal resistance, degradation of complex organic compounds, production of enterotoxins and colicins, and the production of restriction enzymes (16).

This study was undertaken to identify the various Gram-negative bacilli, isolated from patients admitted in Al-Nu'man General Hospital in Baghdad city and to estimate the virulence factor.

Materials and Methods

Specimens

50 sample which analyzed in this study included wound samples consisted 30 and urine samples were 20 , collected from Al-Nu'man General Hospital in Baghdad City. Sampling activates were carried out from 23April to 23June 2014. The age of patient range between 40-70 Years. All the work was done in Microbiology Lab. Department of Biology, College of Education for pure sciences Ibn Al-Haitham, University of Baghdad,

Isolation and identification of gram negative bacteria

Culture

50 Sample were cultured on Blood agar , MacConkey agar and Eosin Methylene Blue (EMB) (17).

Biochemical test

Gram negative bacteria isolated on respective selective and differential media were identified on the basis of colonial, morphological, Gram stain and biochemical tests, IMViC, Urea, Kligler Iron Agar (17) and also used automatically identification system Vitek 2 with GN card (Gram-negative fermenting and non-fermenting bacilli).

Biofilm formation

The Gram-negative isolates ability to colonize abiotic surface was investigated by using Christensen *et al* method (18). The *E. coli* isolates were cultivated in tubes with Trypton soy broth and incubated aerobically at 37^0C for 48 hours and thereafter the culture tubes were emptied carefully and stained with crystal violet solution 1% for 30 minutes, then tubes rinsed with distilled water and left to dry at room temperature. Results were compared with negative control and notice biofilm formation as a layer at the internal wall of tubes by naked eye indicate appositive result.

Hemolysin production

Gram-negative isolates for blood hemolysis production were performed on blood agar plates containing 5% (v/v) human blood and incubated aerobically at 37^0C for 24 hours (19).

β- lactamase production

Iodometric tests was used to detect β- lactamase activity (20).

Antibiotic sensitivity test

Antibiotic susceptibility profiles of Gram-negative isolates was determined by the standard Kirby-Bauer disk diffusion method (21).These antibiotics with their respective disk concentrations are Amoxacillin-clavulanic acid (30µg), Carbenicillin (100µg), Ceftazidime

(30µg), Ampicillin (10µg), Cefalothine (10µg), Cefotaxime (30µg),Imipenem (10µg),Meropenem (10µg). Bacterial cultures suspension equivalent of 0.5 tube McFarland turbidity standards were spread on Muller-Hinton agar plates using sterile swabs and incubated aerobically at 37^0C for 24 hours, then inhibition zones diameter around antibiotic disks were measured. Results were expressed susceptible or resistant according to the criteria recommended by the CLSI (22).

Plasmid DNA isolated procedure

All Gram-negative isolates were screened for plasmid content by the alkaline method of Brinboim and Doly (23). Separated on a 1% agarose, at 50 vol. for 1hr. and 1.30 hr. The DNA bands were visualized and photographed under UV light after the gel had been stained with ethidium bromide.

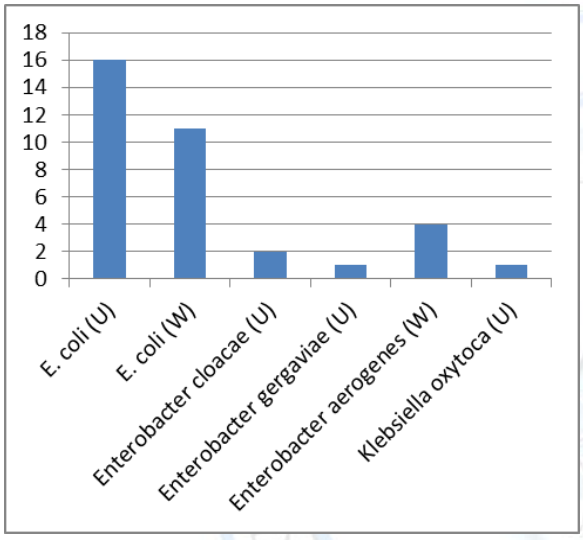
Results and Discussion

The results showed that 50 Gram negative isolates obtained from wound and urine samples were classified into six genera and 9 species (table1). 35/50 isolates (70%) were lactose fermenter and 15/50 isolates (30%) were non lactose fermenter (fig. 1 and 2). Recent study in Nigeria appeared 80.4% were lactose-fermenting members of the *Enterobacteriaceae* and 19.6% were non-lactose fermenting gram-negative bacteria (24). The most common bacterial isolate was *E.coli* (54%), followed by *Proteus vulgaris* (18%), *Enterobacter aerogenes* (8%), *Proteus mirabilis* (6%), *Enterobacter cloacae* (4%), *Acinetobacter baumannii* (4%), and (2%) for each *Klebsiella oxytoca, Enterobacter gergaviae* and *Pseudomonas mendocina.* Study by Panta *etal* in 2013, agreement with our study that showed *E.coli* the major isolates in the different clinical samples (urine and pus) followed by *Salmonella typhi, Klebsiella spp., Salmonella paratyphi, Acinetobacter, Proteus spp.*(3). Other study showed the most prevalent Gram-negative bacteria were *E.coli* (31.6%), *Pseudomonas aeroginosa* (31.2%), *Acinetobacter baumannii* (10.8%), *Klebsiella pneumonia* (8.3%), *Klebsiella spp.* (6.2%), *Haemophilus influenze* (3.7%), *Proteus spp*. (3.3%), and *Enterobacter spp*. (1.9%) (4).

Table 1: Distribution of bacterial species in different clinical samples

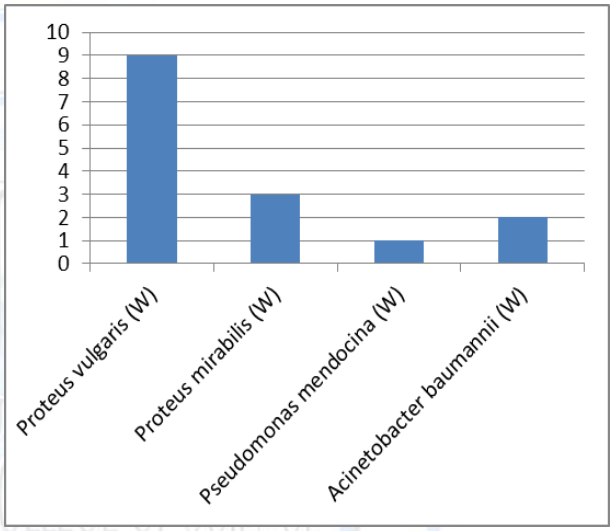
No.	Bacteria species	Urine samples	Wound samples	No. and % of Isolates
1	*Escherichia coli*	16	11	27 (54%)
2	*Enterobacter cloacae*	2	0	2 (4%)
3	*Enterobacter gergaviae*	1	0	1 (2%)
4	*Enterobacter aerogenes*	0	4	4 (8%)
5	*Klebsiella oxytoca*	1	0	1 (2%)

6	*Proteus vulgaris*	0	9	9 (18%)
7	*Proteus mirabilis*	0	3	3 (6%)
8	*Pseudomonas mendocina*	0	1	1 (2%)
9	*Acinetobacter baumannii*	0	2	2 (4%)
Total		20 (40%)	30 (60%)	50 (100%)



*wound W, ** urine U

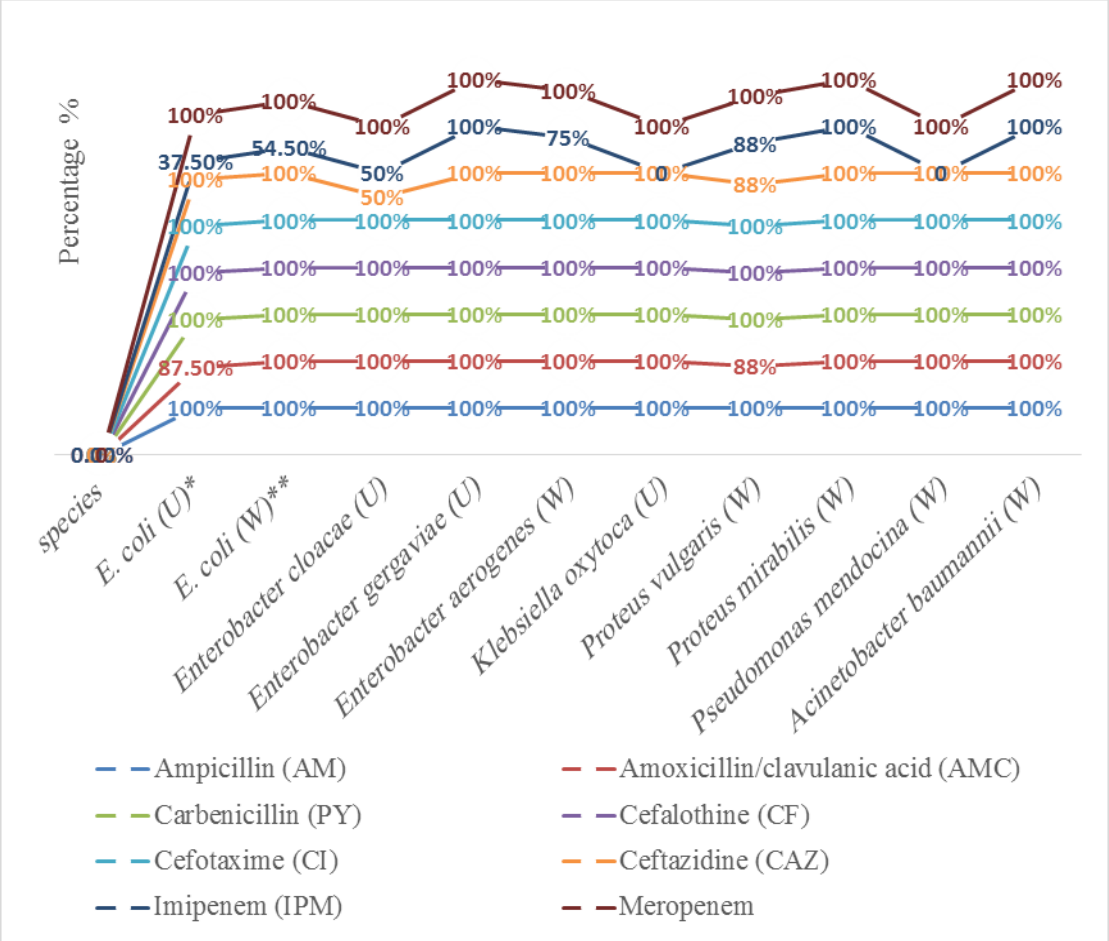
Figure 1. Number of lactose ferment bacteria



*wound W

Figure 2. Number of non- lactose ferment bacteria

Figure 3 showed the sensitivity pattern of the gram negative isolates. All isolates was observed resistance 100% for Ampicillin , Carbenicillin, Cefalothine, Cefotaxim, and Meropenem. This results agreement with previous global and local research.Gram negative bacteria were multi drug resistant to Ampicillin, Amoxicillin, Ceftizoxime. Many studies have showned that active efflux pump or produced beta lactamase enzyme, can be a mechanism of resistance for almost all antibiotics (3, 4, 14, 25, 26).



*urine sample, **wound sample

Figure 3. Resistance percentage of bacterial species isolated from urine and wound samples.

This study appeared that β-lactamase recorded 86% positive result and for β – hemolysis 48% and Biofilm 84%. β-lactamase mainly found in *Escherichia coli, Klebsiella species, Proteus species* but can also occur in other members of Enteroacteriaceae family and in some non enteric organisms such as *Acinetobacter species* , this results agreement with study in USA (27, 28). *Proteus spp*. isolates showed high number of β–hemolysis and *E. coli*. Hemolysin is an extracellular protein toxin which is produced by some strains of *E. coli*, particularly those which cause extraintestinal infections in man (29,30) and also produce by many strain of *Proteus* agreement with (31). Most isolates revaled high activety of biofilm formation 84% of isolates showed biofilm production except *Klebsiella oxytoca*. The ability of bacteria to form biofilms helps them to survive hostile conditions within host and is considered to be responsible for chronic or persistent infections our study agreement with (32).

Table 2.Number of bacterial species produce β-lactamase, β –hemolysis and Biofilm

No.	Bacteria Species	No. of Isolates	β-lactamase	β -hemolysis	Biofilm
1	*Escherichia coli* (U)	16	10	4	10
2	*Escherichia coli* (W)	11	11	4	11
3	*Enterobacter cloacae(U)*	2	2	1	2
4	*Enterobacter gergaviae(U)*	1	1	0	1
5	*Enterobacter aerogenes(W)*	4	4	2	4
6	*Klebsiella oxytoca* (U)	1	1	1	0
7	*Proteus vulgaris (W)*	9	9	7	9
8	*Proteus mirabilis* (W)	3	3	3	2
9	*Pseudomonas mendocina(W)*	1	1	1	1
10	*Acinetobacter baumannii (W)*	2	1	1	2
	Total number (%)	50	43 (86%)	24 (48%)	42 (84%)

There are no evidence of plasmid copy in selected 36 clinical isolates (Figure 4,5) except one isolates of *Proteus vulgaris* (Figure 4/sample number 29) shwod large plasmid (10000 bp). Genetic information can be passed horizontally by transposons, plasmids and bacteriphage: for example when antibiotic resistance genes are carried on plasmids they can be passed between unrelated types of bacteria. Since genes carried on plasmid sometimes incorporated into the chromosome, agene can easily move from one organism to an unrelated one by bacteriophages, plasmids or transposons by conjugation or by transformation (33). In many cases, virulence genes are foundin large contiguous blocks known as chromosomal inserts or pathogenicity islands (34, 35).

In conclusion, our study revealed that *Escherichia coli* was the most common followed by *Proteus vulgaris*. one isolates of *Proteus vulgaris* showed large plasmid (10000 bp) in size.

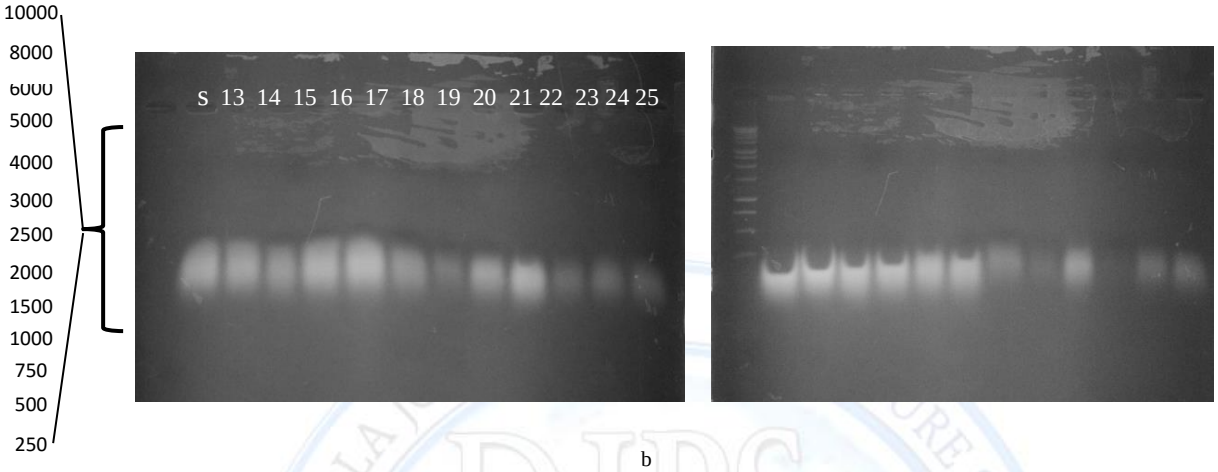


Figure 4: Agarose gel electrophoresis of plasmids extracted from various clinical sample: a- sample 1-12 b-sample 13- 24. S. 1kb(250-10000bp) DNA ladder (Promega); (1% agarose, 50 vol. 1.30 hours).

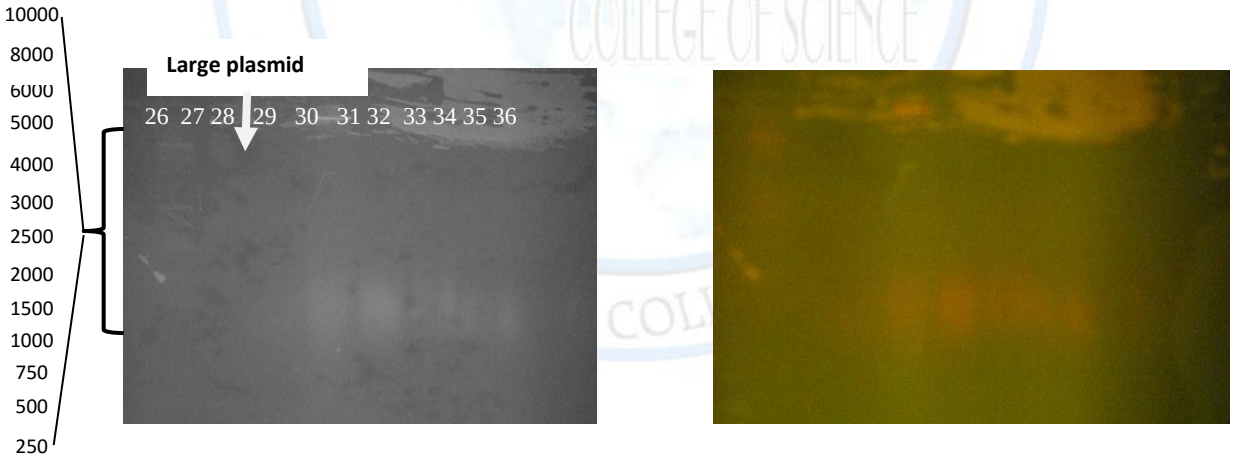


Figure 5: Agarose gel electrophoresis of plasmids extracted from various clinical sample: a- sample 25-36b-negatve picture. S. 1kb(250-10000bp) DNA ladder (Promega); (1% agarose, 50 vol. 1.30 hours).

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