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ß-LACTAMASE PRODUCTION AND ANTIMICROBIAL RESISTANCES BY *KLEBSIELLA SPECIES* ISOLATED FROM DIARRHEAL CASES AMONG CHILDREN IN KIRKUK CITY - IRAQ

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ABSTRACT The study included the isolation and identification of 93 *Klebsiella* species (*spp.)* from 454 children suffering from acute diarrhea. The rapid standard iodometric method was used for the detection of ß-lactamase production. The Results showed that 49/93 (52.7%) *Klebsiella* isolates were positive for ß-lactamase production. *Klebsiella spp*. that showed positive result by using iodometric method, they had been tested with nitrocefin disk and spot iodometric method (filter paper method) as confirmative tests, whereby positive results were obtained by using these two methods as well. In the present study, 28/49 (57.1%) isolates appeared belonging to *K. pneumoniae*, 10/49 (20.4%) isolates belonging to *K. oxytoca*, 10/49 (20.4%) isolates belonging to *K. terrigena* and 1/49 (2%) isolates belonging to *K. ornithinolytica*.

Antibiotic sensitivity test was performed to the 49 ß-Lactamase producer using 30 different antibiotics. It was obvious that Imipenem is more active against *Klebsiella spp*. followed by ciprofloxacin, norfloxacin, amikacin and cefoxitin, and their resistance were (2.1%), (2.0%), (4.1%), (4.1%) and (12.2%) respectively, and all isolates showed multidrug resistance against 10 antibiotics at least. Results showed that all the isolate (100%) were able to resist ampicillin, amoxicillin, clindamycine, rifampicin, and vancomycin; while they were highly resistant to amoxicillin (97.9%), erythromycin (97.9%), carbencillin (95.9%), cephalothin (91.8%), cephradin (79.6%), cephalexin (75.5%), and pipracillin (69.4%).

KEYWORDS : Acute diarrhea, Klebsiella, ß-lactamase, Antibiotic sensitivity, Kirkuk

Introduction:

Klebsiella spp. are gram-negative rods belonging to the family *Enterobacteriaceae* [1, 2]. The genus *Klebsiella* comprises five species, *K. pneumoniae*, *K. oxytoca*, *K. planticola*, *K. terrigena* and *K. ornithinolytica* [3]. *Klebsiella spp.*, particularly *K. pneumoniae*, is an important cause of nosocomial infections, and the main population at risk is neonates and immunocompromised hosts. Outbreaks of multiple resistant *Klebsiella*, which caused systemic infections and death, were widely reported [4]. *K. pneumoniae* is frequently found in many different geographical locations, with beta-lactamase resistance becoming a growing problem [5]. The wide spread use of antimicrobial agents in the treatment of infections has led to serious problems of antimicrobial resistance. The emergence and spread of antibiotic resistance in bacteria of medical importance imposes serious constraint on the option available for treatment of many infections [6]. Li and Lim mentioned that in the last 10 years, the extensive spread of multiple antibiotic –resistant *K. pneumoniae* strains has become a major threat to the ever-increasing number of immunocompromised patients [7].

The aims of this study are to investigate the occurrence of *Klebsiella* spp. isolates in human stool samples from children suffering from diarrhea in Kirkuk-city, and evaluating the susceptibility of ß-lactamase producer isolates to some antibiotics.

Materials and Methods:

Sample collection

The study was carried out on children (outpatients as well as inpatients suffering from acute diarrhoea) attended Azadi teaching hospital and Pediatric hospital in Kirkuk city, from June 2009 to June 2011, where a total of (454) children submitted to the study under physician supervision. Stool specimens were collected in disposable, clean screw-capped, commercially available containers used for this purpose. All specimens were processed immediately or were kept using Carry Blair transport media in case of delay for 1-2 hours after their collection, and to be cultured thereafter [8].

Bacterial isolation and identification:

Collected samples were cultured directly on MacConkey agar; Xylose Lysine deoxycholate (XLD) agar, Salmonella Shigella (SS) agar and Eosin Methylene Blue (EMB) agar for primary isolation of the Enterobacteriaceae and blood agar to detect beta hemolytic isolate [8]. All isolates incubated aerobically at 35 ˚C for 24 hours, and select suspicious colonies for definitive microscopic examination, culture characteristics, biochemical testing and the usage of API 20E System (BioMérieux/France) for identifying Enterobacteriaceae and other gram negative rods [9, 8, 10, 11, 12, 13, 14].

Detection of β-lactamase by three methods:

1. Rapid iodometric method [15]
2. Spot iodometric method (Filter paper method) [16]
3. Cefinase test -Nitrocefin disk/Fluka [17, 13]

Antibiotic susceptibility test by disk diffusion method (Kirby Bauer test)

This method is one of the best standardized tests and that its performance is continually updated by the National Committee for Clinical Laboratory Standards (NCCLS) consensus effort. The test was performed as follow.

- **Preparing of inoculum and inoculation of test plate and disc placement**

Inoculums from the tested bacterium was prepared. A single colony was transferred to a fresh test tube contained 5ml nutrient broth then incubated at 37 ˚C for 24 hr. to prepare inoculums at a log-phase. After comparing and adjusting with McFarland tube number 0.5, a sterile cotton swab is dipped into the inoculum and then swabbed evenly across the surface of a Muller-Hinton agar plate. Within the following 15 minutes after inoculation, the antimicrobial-containing disks (Table-1) are applied to the agar with a forceps pressed firmly to ensure contact with agar and then plate inverted and incubated at 37 ˚C for 18 hours.

- **Reading the results**

After incubation, the diameter of each inhibition zone was measured by millimeter (mm), and the isolate was interpreted as either susceptible, intermediate, or resistant to a particular drug by comparison with standards inhibition zone [18]. The diameters of inhibition zones around the antibiotic disks were measured and compared with the standard manual recommended by the national

committee for clinical laboratory standards (NCCLs) guideline (Table-1).

Table-1: Antibiotic disc used in the present study

Antibiotics	Symbol	Concentration µg/disc	Inhibition zone /mm			Manufacturer
			R	I	S	
Penicillin						
Amoxicillin	AX	25	≤13	14-16	≥17	Bioanalysed/ England
Ampicillin	AM	10	≤13	14-16	≥17	=
Pipracillin	PRL	100	≤17	18-20	≥21	=
Amoxicillin + Clavulanic Acid	AMC	30	≤13	14-17	≥18	=
Carbencillin	CB	100	≤19	18-22	≥23	=
Cephalosporins						
Cephalexin	CL	30	≤14	17-15	≥18	=
Cephalothin	CF	30	≤14	17-15	≥18	=
Cephradin	CE	30	≤14	17-15	≥18	=
Cefoxitin	FOX	30	≤14	17-15	≥18	=
Ceftriaxone	CRO	30	≤19	20-22	≥23	=
Cefotaxime	CTX	30	≤14	22-15	≥23	=
Ceftazidime	CAZ	30	≤17	18-20	≥21	=
Cefixime	CFM	5	≤15	16-18	≥19	=
Carbapenems						
Imipenem	IPM	10	≤19	20-22	≥23	=
Aminoglycosides						
Gentamicin	GM	10	≤12	13-14	≥12	=
Amikacin	AK	30	≤14	15-16	≥17	=
Tobramycin	TOB	10	≤12	13-14	≥15	=
Macrolides						
Erythromycin	E	10	≤20	28-21	≥29	=
Azithromycin	AZM	15	≤13	17-14	≥23	=
Lincosamide						
Rifampicin	RA	10	≤8	-	≥8	=
Trimethoprim	TMP	5	≤10	11-15	≥16	=
Quinolones						
Vancomycin	VA	30	≤9	10-11	≥12	=
Ciprofloxacin	CIP	5	≤15	16-20	≥21	=
Chloramphenicol	C	30	≤12	17-13	≥18	=
Clindamycine	CA	15	≤14	16-15	≥21	=
Nalidixic Acid	NA	30	≤13	18-14	≥19	=
Nitrofurantoin	F	300	≤14	15-17	≥17	=
Norfloxacin	NOR	10	≤12	13-16	≥17	=
Tetracyclines						
Doxycycline	DA	2	≤12	13-15	≥16	=
Tetracycline	TE	30	≤14	15-18	≥19	=

Results

Isolation and identification:

The results showed that among the 454 cultured stool sample, 433 samples revealed positive cultures and 767 bacteria have been isolated from children with diarrhea. Ninety three (93, 12.13%) *Klebsiella spp*. isolates were obtained from the positive stool samples.

Detection of ß-lactamase production

Table-2 confirmed that 49 out of 93 (52.7%) *Klebsiella* isolates had positive results for ß-lactamase production, which had been shown using rapid iodometric method. The table shows the percentages of different *Klebsiella* species which produced ß-lactamase (namely, *K. pneumonia, K. oxytoca*, *K. terrigena, K. ornithinolytica*). In the other hand, it is clear that some of these strains were negative for ß-lactamase production as it is listed in the table.

Table-2: β lactamase result of *Klebsiella spp.*

Isolates / Characters	K. pneumoniae		K. oxytoca		K. terrigena		K. ornithinolytica		Total
	No	%	No	%	No	%	No	%	
-lactamase +ve	28	45.9 %	10	55.6 %	10	83.3 %	1	50%	49
-lactamase-ve	33	54.1 %	8	44.4 %	2	16.7 %	1	50%	44
Total (93)	61	100%	18	100%	12	100%	2	100%	93

All *Klebsiella* strains that had positive result by iodometric method were furthermore tested with nitrocephin disk and spot iodometric method as confirmative tests, where they showed positive result by these two methods (Figure-1). The remaining 44 isolates (47.3%) failed to produce ß-lactamase enzyme (no color change was detected by iodometric method).

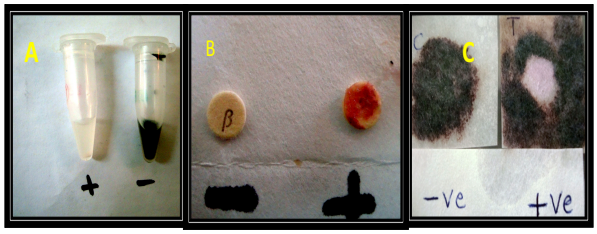

Figure-1: ß-Lactamase detection: A- rapid Iodometric method, B- Nitrocefin disks, and C- filter paper methods.

Resistant to antibiotics:

All 49 ß-lactamase producing *Klebsiella* isolates were tested for their antibiotic resistance against 30 of antimicrobial agents using disk diffusion method.

The antibiotics were represented by ß-lactams, aminoglycosides, Lincosamide, quinolones, tetracyclines, and others. All isolates were found to be resistant to at least different 10 antibiotics to which they were tested, hence all the isolates were considered as multidrug resistant.

Susceptibility tests were performed for the 49 -lactamase producer *Klebsiella spp.* (28 isolates as *K. pneumoniae*, 10 as *K. oxytoca*, 10 as *K. terrigena*, and 1 as *K. ornithinolytica*). The results in Table-3 and Table-4 show that all *those Klebsiella spp.* are resistant to ampicillin (100%), amoxicillin + clavulanic acid 100%, clindamycine 100%, rifampicin 100%, vancomycin 100%, metronidazole 100%, amoxicillin 97.9%, erythromycin 97.9%, carbencillin 95.9%, cephalothin 91.8%, Cephradin 79.6%, Cephalexin 75.5%, pipracillin 69.4%, tetracycline 51%, trimethoprim 49%, cefotaxime 49%, Ceftriaxone 36.7%, ceftazidime 36.7%, cefixime 30.6%, nitrofurantion 18.4%, gentamicin 18.4%, tobramycin 14.3%, azithromycin 14.3%, chloramphenicol 14.3%, cefoxitin 12.2%, nalidixic acid 12.2%, amikacin 4.1%, norfloxacin 4.1%, Ciprofloxacin 2.1% and Imipenem 2.1%.

It was also clear from Table-4 that 34/49 (69.4%) of isolates were resistant to piperacillin.

The results also revealed high resistance of *Klebsiella* isolates to first generation cephalosporins; wereby 39/49 (79.6%) of *Klebsiella* isolates were resistant to Cephradin, 37/49 (75.5%) were resistant to Cephalexin and 45/49 (91.8%) were resistant to Cephalothin, but they showed low level of resistance (6/49; 12.2%) to Cefoxitin, a second generation member.

This study clarified that about half of *Klebsiellae* isolates were resistant to third generation cephalosporins (3GC). Cefotaxime resistance was seen in 24 out of 49 (49%) of *Klebsiella* isolates, but they showed moderate to low level of resistance to ceftazidime in 18 out of 49 (36.7%) of *Klebsiella* isolates, ceftriaxone (18/49; 36.7%) and cefixime (15/49; 30.6%).

The results revealed that among the 49 of *Klebsiellae* isolates, 48 (97.9%) were sensitive to carbapenems (imipenem) (Table-3). The resistance to aminoglycosides were recorded in 9 (18.4%), 2 (4.1%), and 7 (14.3%) isolates to gentamycin, amikacin, and tobramycin respectively, versus 0 (0%), 5 (10.2%), 5(11.6%) and 2 (4.1%) isolates were intermediately resistant to antibiotics mentioned above respectively.

Table-3: Antibiotic susceptibility of β - lactamase producer *Klebsiella spp.*

Isolates / Antibiotic – Symbol			Klebsiella spp. (49) S	I	R
Penicillin					
1	Amoxicillin	AX	0 (0%)	1 (2.0%)	48 (97.9%)
2	Ampicillin	AM	0 (0%)	0 (0%)	49 (100%)
3	Pipracillin	PRL	8 (16.3%)	7 (14.3%)	34 (69.4)
4	Amoxicillin + Clavulanic acid	AMC	0 (0%)	0 (0%)	49 (100%)
5	Carbencillin	CB	2 (4.1)	0 (0%)	47 (95.9%)
Cephalosporins					
6	Cephalexin	CL	6 (12.2%)	6 (12.2%)	37 (75.5%)
7	Cephalothin	CF	3 (6.1%)	1 (2.0%)	45 (91.8%)
8	Cephradin	CE	3 (6.1%)	7 (14.3%)	39 (79.6%)
9	Cefoxitin	FOX	39 (79.6%)	4 (8.2%)	6 (12.2%)
10	Ceftriaxone	CRO	22 (44.9)	9 (18.4%)	18 (36.7%)
11	Cefotaxime	CTX	13 (26.5%)	12 (24.5%)	24 (49%)
12	Ceftazidime	CAZ	29 (59.2%)	2 (4.1)	18 (36.7%)
13	Cefixime	CFM	33 (67.3%)	1 (2.0%)	15 (30.6%)
Carbapenems					
14	Imipenem	IMP	48 (97.9%)	0 (0%)	1 (2.1%)
Aminoglycosides					
15	Gentamicin	GM	40 (81.6%)	0 (0%)	9 (18.4%)
16	Amikacin	AK	42 (85.7%)	5 (10.2%)	2 (4.1)
17	Tobramycin	TOB	40 (81.6%)	2 (4.1)	7 (14.3%)
Macrolides					
18	Erythromycin	E	1 (2.0%)	0 (0%)	48 (97.9%)
19	Azithromycin	AZM	37 (75.5%)	5 (10.2%)	7 (14.3%)
Lincosamide					
20	Rifampicin	RA	0 (0%)	0 (0%)	49 (100%)
21	Trimethoprim	TMP	24 (49%)	1 (2.0%)	24 (49%)
22	Vancomycin	VA	0 (0%)	0 (0%)	49 (100%)
23	Ciprofloxacin	CIP	46 (93.9)	2 (4.1)	1 (2.0%)
24	Chloramphenicol	C	41 (83.7%)	1 (2.0%)	7 (14.3%)
25	Clindamycine	CA	0 (0%)	0 (0%)	49 (100%)
26	Nalidixic Acid	NA	40 (81.6%)	3 (6.1%)	6 (12.2%)
27	Nitrofurantoin	F	29 (59.2%)	11 (2204%)	9 (18.4%)
28	Norfloxacin	NOR	46 (93.9%)	1 (2.0%)	2 (4.1)
Tetracyclines					
29	Doxycycline	DA	0 (0%)	0 (0%)	49 (100%)
30	Tetracycline	TE	14 (28.6)	10 (20.4%)	25 (51%)

Table-4: Antibiotic resistance of β -lactamase producer *Klebsiella spp.*

Isolates / Antibiotic - Symbol			K. pneumoniae (28) No	%	K. oxytoca (10) No	%	K terrigena (10) No	%	K. ornithinolytica (1) No	%
1	Amoxicillin	AX	28	100 %	10	100 %	9	90%	1	100 %
2	Ampicillin	AM	28	100 %	10	100 %	10	100 %	1	100 %
3	Pipracillin	PRL	21	75%	6	60%	6	60%	1	100 %
4	Amoxicillin + Clavulanic acid	AMC	28	100 %	10	100 %	10	100 %	1	100 %
5	Carbencillin	CB	26	92.9 %	10	100 %	10	100 %	1	100 %
6	Cephalexin	CL	22	78.6 %	9	90%	6	60%	0	0%
7	Cephalothin	CF	25	89.3 %	10	100 %	10	100 %	0	0%
8	Cephradin	CE	23	82.1 %	9	90%	7	70%	0	0%
9	Cefoxitin	FOX	4	14.3 %	0	0%	2	20%	0	0%
10	Ceftriaxone	CRO	15	53.6 %	1	10%	2	20%	0	0%
11	Cefotaxime	CTX	19	67.9 %	2	20%	3	30%	0	0%
12	Ceftazidime	CAZ	11	39.3 %	3	30%	3	30%	0	0%
13	Cefixime	CFM	12	40.7 4%	1	10%	2	20%	0	0%
14	Imipenem	IMP	1	3.7 %	0	0%	0	0%	0	0%
15	Gentamicin	GM	6	18.5 %	2	20%	1	10%	0	0%
16	Amikacin	AK	1	3.7 %	0	0%	1	10%	0	0%
17	Tobramycin	TOB	5	18.5 %	1	10%	1	10%	0	0%
18	Erythromycin	E	28	100 %	10	100 %	10	100 %	0	0%
19	Azithromycin	AZM	5	18.5 %	1	10%	1	10%	0	0%
20	Rifampicin	RA	28	100 %	10	100 %	10	100 %	1	100 %
21	Trimethoprim	TMP	18	62.9 6%	1	10%	5	50%	0	0%
22	Vancomycin	VA	28	100 %	10	100 %	10	100 %	1	100 %
23	Ciprofloxacin	CIP	1	3.7 %	0	0%	0	0%	0	0%
24	Chloramphenicol	C	5	14.8 %	1	10%	1	10%	0	0%
25	Clindamycine	CA	28	100 %	10	100 %	10	100 %	1	100 %
26	Nalidixic Acid	NA	5	14.8 %	1	10%	0	0%	0	0%
27	Norfloxacin	NOR	1	3.7 %	0	0%	1	10%	0	0%
28	Nitrofurantion	F	6	18.5 %	1	10%	2	20%	١	100 %
29	Doxycycline	DA	28	100 %	10	100 %	10	100 %	1	100 %
30	Tetracycline	TE	15	51.8 5%	4	40%	6	60%	0	0%

The resistance pattern of *Klebsiellae* isolates to tetracycline and ciprofloxacin was represented by 25/49 (51%) and 1/49 (2.0%) isolates respectively, while 10/49 (20.4%) and 2/49 (4.1%) of isolates gave intermediate resistance to those antibiotics.

The results of the present study have shown that 24/49 (49%) of *Klebsiellae spp*. isolates were resistant to trimethoprim which is a member of sulfonamides.

The present study recorded that 7/49 (14.3%) of *Klebsiella* isolates were resistant to chloramphenicole while 1/49 (2.0%) were intermediately resistant to it.

L-Form formation by *Klebsiella spp.*

Some *Klebsiella* isolates exhibited L- form around some antibiotics (Table-5 and Figure-2) during the process of antibiotic sensitivity test, and they recovered their original shape when subcultured on MacConkey agar and Blood agar media. They also showed the same biochemical reaction when reidentified except lacking the mucoid colony. Both Nalidixic Acid (NA) and trimethoprim (TMP) caused L-form formation more than other antibiotics.

Table-5: L- Form production by *Klebsiella spp.*

Isolates name		Isolates NO.	Antibiotics induced l-form formation
1	K. pneumoniae	129	CFM, CRO, TMP, CTX, PRL, ATM
2	K. pneumoniae	130	TMP
3	K. pneumoniae	131	NA, CFM, CTX, PY
4	K. pneumoniae	140	NA
5	K. pneumoniae	292	NA
6	K. pneumoniae	307	TOB

7	K. pneumoniae	486	NA, CTX, CRO
8	K. pneumoniae	488	NA, AFM
9	K. oxytoca	467	TMP, FOX
10	K terrigena	63	TMP, CAZ, PRL, CTX
11	K. terrigena	67	TMP
12	K. terrigena	93	NA, TMP
13	K. terrigena	256	TMP, CFM, ATM, NA, CRO, TE, FOX, CL, CE
14	K. terrigena	324	NA, ATM

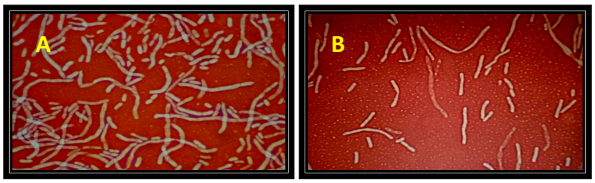

Figure-2: L- Form production by *Klebsiella spp.* A: *K. pneumoniae* isolate number 129. B: *K. terrigena* isolate number 256.

Discussion

In general, some pathogens are responsible for diarrhea in infants and young children worldwide; and the frequency and proportion of the specific diarrhoeal pathogens identified may be different in different places and laboratories [19]. In recent years, many studies were conducted to evaluate the role of agent causing gastroenteritis previously thought to be mere commensals of the gastrointestinal tract [20], several cases of diarrhea due to *Hafnia alvei* [21, 22], *Citrobacter freundii* [23, 24], *Enterobacter aerogenes* [25], *Morganella morganii* [26], *Providencia alcalifaciens* [27, 28], *Proteus mirabilis* [29], *Pseudomonas aeruginosa* [30,31] and *Klebsiella spp*. [32, 33] have been reported.

The current work declared that *Klebsiella spp*. composed 12.13% of the bacteria isolated from the positive cultures of the stool samples. A result which does not differ from the reports mentioned above.

As a cause of nosocomial gram-negative bacteremia, *Klebsiella* is second only to Escherichia coli [34, 35, 36, 37] in pediatric wards [38]. *Klebsiella pneumoniae* and *Klebsiella oxytoca*, both commensals of the human gastrointestinal tract, have been reported to be an occasional cause of diarrhea in humans [39]. *K. pneumoniae* is found in the intestinal flora of healthy individuals, but usually in small numbers [40]. In the tropics, however, strains of *K. pneumoniae* have been isolated in high numbers from the small bowel of persons with acute diarrhoea [41, 42] and malnourished children who have chronic diarrhoea [43, 44].

Regarding the enzymatic activity, the current work indicated that production of ß-lactamase was prevalent in more than half (52.7%) of *Klebsiella* isolates. The remaining (47.3%) failed to produce ß-lactamase enzyme. A negative ß-lactamase test result does not rule out resistance due to other mechanisms, like decreased permeability or decreased affinity of the target PBPs particularly in *Klebsiella* [45]. The other reason may be due to production of low quantities of the enzyme in the periplasmic space, making its detection is more difficult [46].

Rapid iodometric method was used for detection of ß-lactamase production in *Klebsiella* strains. This method depends on detection of penicilloic or cephalospoic acid, resulted from breakdown of amide bond in ß-lactam ring for each of penicillins or cephalosporins (46, 47). It was found that the rapid reaction iodine into iodide is based on the concentration of the released penicillinase or cephalosporinase enzyme. Factors such as temperature and pH also play an important role in enhancement or reduction of enzyme activity [48].

Iodine reacts with starch for formation of dark blue complex, which stay without changing in the absence of ß-Lactamase enzyme (Figure-1: A). In the case of ß-Lactamase producing bacteria, the resulting penicilloic or cephalospoic acid will reduce iodine into iodide; consequently, decolorization of starch-iodine complex occurs (changing the color directly to white) of an isolate is a ß-lactamase producer but not if the enzyme is absent [49].

The rapid iodometric assay was characterized by; simplicity to perform it, short period for yielding results, and availability of materials required for achieving the test [50, 51].

While spot iodometric method (Figure-1: C) has several useful features; the reaction is completed within 10 min. There is no need to prepare reagents or to adjust pH, and Gram iodine is readily available in most microbiology laboratories. In addition, the penicillin powder is inexpensive and stable in dry form for at least 6 months or more. It is particularly useful when the tests are performed infrequently [16].

The more sensitive technique is to measure ß-Lactamase activity with a chromogenic cephalosporin, usually nitrocephin [52] which changes from yellow to pink/red on hydrolysis. In addition, for many ß-lactamases, nitrocephin is the substrate that is most readily hydrolyzed by the enzyme. This property makes it often the most sensitive detection system and it provides a very rapid and continent method for detection of ß-lactamases. nitrocefin can be used as a solution or as disks (Figure-1: B) upon which test cultures are smeared [46].

The iodometric method is cheaper than nitrocephin and, given care, almost as sensitive, but it might give false-positive results, which probably reflect non specific reaction of iodine with bacterial proteins after one hour of reaction. However, this disadvantage has been eliminated and the test has been improved in the present study by reducing the time of the reaction to 5 min., thus only rapid ß-lactamase producers can be detected.

Other disadvantage of this method is that the low levels of inducible chromosomal ß-Lactamases are often inadequate to give a color reaction. However, *Klebsiella* ß-lactamases differ greatly from the class C enzymes of many gram-negative rods such as *Enterobacter spp.*, *Morganella morganii*, *Providencia stuartii*, *P. rettgeri*, and *Pseudomonas spp*., which typically have inducible expression of these enzymes [45, 53, 54, 55, 56, 57].

Increase in the colonization rates with *Klebsiella* was observed and it occurred primarily in patients receiving broad spectrum or multiple antibiotics [58], furthermore, widespread use of antimicrobial therapy has often been responsible for the occurrence of multiply resistant *Klebsiella* strains in hospitals [59, 60].

The high resistance of all *Klebsiella spp.* to ampicillin 100%, amoxicillin + clavulanic acid 100%, amoxicillin 97.9% and carbencillin 95.9%, as it is clear in this study, was related to different mechanisms which mediate antibiotic resistance in *Klebsiellae* that were recorded by other local investigators, Al-Saedi, who observed *K. pneumoniae* (98.2%) resistance to amoxicillin, and Darweesh who observed that all isolated *Klebsiella spp.* from different clinical specimens showed high resistance (100%) against ampicillin [61, 62]. Lucet *et al*., demonstrated that *Klebsiellae* can resist cephalosporins, penicillins and other ß-lactamase via alternating the permeability of plasma membrane preventing antibiotics from entry to the bacterial cell, and also it was found that the major mechanism of resistance in gram negative bacteria causing clinically significant infection is the expression of ß-lactamases, of which there are several classes including plasmid encoded and chromosomally encoded enzymes [63]. The emergence of chromosomally encoded resistance to ampicillin, and carbenicillin has been reported by many studies, which also found that the ß-lactamase enzyme SHV-1 was widely distributed among *Klebsiella spp.* conferring resistance to these ß-Lactam antibiotics [47, 64].

We found that more than two thirds of isolates were resistant to piperacillin. Although this antibiotic was first introduced in clinical therapy in 1978 [65], the low level of resistance to this drug as compared to other ß-Lactam antibiotics may be attributed to the fact that it was recently introduced into Iraqi market.

As the cephalosporins are concerned, the high resistance of *Klebsiella* isolates to first and second generations cephalosporins was made clear in this work. These results are in agreement with results reported by other researchers who found that chromosomally encoded resistance to first and second generation cephalosporins in strains of *Klebsiella* has been emerged in many hospitals [47, 66].

Besides, about half of *Klebsiellae* isolates showed resistance to third generation cephalosporins (3GC), whereby 49% of *Klebsiella* isolates were Cefotaxime resistant, but they showed moderate to low level of

resistance to ceftazidime in 36.7% of *Klebsiella* isolates, ceftriaxone in 36.7% and cefixime in 30.6%. This is consistent with number of reports. Ceftazidime and cefotaxime resistances are markers for the presence of extended spectrum ß-lactamases (ESBLs). Resistance of *Klebsiella spp.* to 3GC has been reported by many researchers. Transferable resistance to cefotaxime was demonstrated in *K. pneumoniae* and it was found that the enzyme responsible was SHV-2 ß-lactamase [67]. In addition, plasmid mediated ß-Lactamases with high hydrolytic activity against cefotaxime was also reported in strains of *K. pneumoniae* [68]. Although ceftazidime is remarkably resistant to the activities of most ß-lactamases, its use has been associated with the isolation of *Klebsiella* strains which elaborate plasmid mediated enzymes with a high degree of activity against this agent [69].

In this study, resistance to other antimicrobial agents was studied. The results revealed that 97.9% of *Klebsiellae* isolates were sensitive to carbapenems (imipenem). This result goes in agreement with the result of Iroha *et al*. [70].

The results illustrated that aminoglycosides are more active against *Klebsiella spp*. Gentamycin resistance was (18.4%), a result which disagrees with the results recorded by Iroha *et al.,* which were (92.6%) and with that of Al-Shamarti which were 25 (58%) [70, 71].

It was obvious that ciprofloxacin is more active against *Klebsiella spp*. A result which goes with with Granier *et al*., who recorded that 60% of *Klebsiella spp.* isolates were susceptible to ciprofloxacin [72]; and Bonnet, who recorded that 68.8% of *K. pneumoniae* were susceptible to ciprofloxacin [73].

About half of the *Klebsiellae* spp. isolates showed resistance to trimethoprim. This finding disagree with the result reported by Iroha *et al.* and Al-Shamarti who found that 79% and 83.7% of *K. pneumoniae* isolates were resistant to trimethoprim [70, 71].

When resistance to chloramphenicole was examined, the results of this work were lower than what was recorded by Al-Shammarti, in which there were as 32.5% showed resistance and 4.6% showed intermediate resistance. Disagreement in antibiotic resistance rates with other studies could be related to the differences in source of samples, places and time of performing the study [71].

Furthermore, L phase variants (L forms) are wall-defective microbial forms that can replicate serially as nonrigid cells and produce colonies on solid media. Some L phase variants are stable; others are unstable and revert to bacterial parental forms. Wall-defective forms are not genetically related to mycoplasmas. They can result from spontaneous mutation or from the effects of chemicals. Treatment of eubacteria with cell wall-inhibiting drugs or lysozyme can produce cell wall-defective microbial forms. Protoplasts are such forms usually derived from gram-positive organisms; they are osmotically fragile, with external surfaces free of cell wall constituents.

Spheroplasts are cell wall-defective forms usually derived from gram-negative bacteria; they retain some outer membrane material. Cell wall-defective forms continue to synthesize some antigens that are normally located in the cell wall of the parent bacteria. Reversion of L forms to the parental bacterial form is enhanced by growth in the presence of 15–30% gelatin or 2.5% agar. Reversion is inhibited by inhibitors of protein synthesis. It is uncertain whether cell wall-defective microbial forms cause tissue reactions resulting in disease. They may be important for the persistence of microorganisms in tissue and recurrence of infection after antimicrobial treatment, as in rare cases of endocarditis [14].

In the present study, both Nalidixic Acid (NA) and trimethoprim (TMP) caused L- form formation more than other antibiotics. This may be resulted from frequent usage of these two antibiotics specially in UTI treatment. Besides, these two antibiotics are bacteriostatic not bactericidal.

Conclusions:

1. A remarkable percentage of *Klebseilla spp.* produce ß-lactamase.
2. All β-lactamase producer *Klebseilla spp.* were multidrug resistant.
3. Meropenem and imipenem are the drugs of choice to treat infections caused by extended spectrum ß-lactamases producing *Klebsiella spp.*
4. Nalidixic Acid (NA) and trimethoprim (TMP) caused L- form formation more than other antibiotics.

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