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Epidemiological studies using biotyping, serotyping and bacteriocin typing as marker systems to identify clinical multidrug-resistant *Klebsiella* spp

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Abstract: **Objective: To assess the efficiency of combined different epidemiological marker systems for identifying and typing clinical multidrug-resistant *Klebsiella* spp. isolates according to biotyping, serotyping, its sensitivity & resistance to some antibiotics and bacteriocin typing as well as relation with spreading as nosocomial infection to improve the management of such outbreaks.**

Methods: In this study one hundred-and four (104) Klebsiella strains were collected. They were selected from 1007 hospitalized patients at Sohag University Hospital. All Klebsiella isolates were subjected to susceptibility testing by disc diffusion method. Serotyping of *Klebsiella spp.* by slide agglutination test using the specific polyvalent antiserum (specific to 38 K-types) used. Monoclonal anti-Klebsiella. IgG2a was the immunoglobulin type. Detection of bacteriocin sensitivity and bacteriocin typing was estimated by the inhibition pattern they form, against 8 reference bacteriocin producer strains using scrap and streak method.

Results: All isolates exhibited multiresistant patterns ranging from 4-12 antibiotics. 99 (95.1%) out of (104) tested strains of Klebsiella isolates shown positive agglutination reaction with polyvalent antibody resembling *K. pneumoniae* serotype. It was observed that most of the isolated strains of Klebsiella were sensitive to more than one of the bacteriocin producer strains 87 (83.6%). {P1}& {P8} bacteriocin producer strains have the broad spectrum of activity. Bacteriocin (Klebocin) typing of Klebsiella strains showed that 21 different combinations of bacteriocin sensitivity patterns were observed. Bacteriocin types {1}, {1,8},{1,3,8},and {1,6,8} were the most prevalent bacteriocin types.

Conclusion: Typing of multiresistant strains can possess a significant contribution in epidemiological studies. The combination of phenotypic markers such as biotyping and serotyping and bacteriocin typing was more discriminating than either method alone.

Keywords: **bacteriocin, Klebsiella, Epidemiological studies.**

1. INTRODUCTION

The genus Klebsiella is widely distributed in nature and may also be a resident in the human bowel. Occasionally, these organisms invade the urinary tract, wounds and blood stream and cause serious infections [(1), (2)]. Nowadays, pyogenic liver abscess (PLA) is still a common and severe intra-abdominal infection, and *Klebsiella pneumoniae* had emerged as the most common pathogenic bacteria worldwide in the past ten years[(3)]. Determining whether resistant bacteria are hospital acquired is often problematic since patients may be colonized asymptomatically when they enter the hospital. The early diagnosis of many of these infections is very important [(4).] Microbiologically resistant organisms are those that possess any resistance mechanism [(5)]. The increasing use of broader-spectrum cephalosporins in the first half of the (1990s) has become one of the major factors responsible for the high rate of selection of (ESBL)- producing microorganisms. This outbreak was due to both plasmid dissemination among unrelated strains and clonal spread of some strains in several

wards of the hospital [5], [6]. Multidrug resistant *K. pneumoniae* is one of the most important causes of nosocomial infection worldwide[7]. One of the main virulence factors of *K. pneumonia*e is its capsular polysaccharide (CPS). CPS is expressed in vivo, promotes biofilm formation, and exerts an anti-opsonic effect, all of which evade the host immune response [4], [8]. The degree of virulence conferred by a particular K-antigen might be connected to the mannose content of the (CPS) [9], [10], [11]. Its massive layer capsule protects the bacterium from phagocytosis [12] by polymorphonuclear granulocytes and prevents bacterial death caused by bactericidal serum factors [13]. Serotyping is currently the most widely used technique for typing *Klebsiella* spp. according to capsular antigens [11], [14], [15]. It shows good reproducibility and is capable of differentiating most clinical isolates [14]. Also, it can be classified into 77 serological types [10], [11], [16]. Bacteriocins are naturally occurring antibacterial substances elaborated by members of the family Enterobacteriaceae and some other bacteria. Many Klebsiella strains produce bacteriocins. Epidemiological analysis may be improved by the use of bacteriocins as an adjunct to capsular serotyping [4], [17]. Bacteriocins are proteinaceous compounds that are antagonistic against bacteria closely related to the producer bacterium [18]. Recent studies demonstrated that the high specificity and activity of klebocin produced by *K. pneumoniae* subspecies pneumoniae against the target cells in contrast of other Gram negative narrow target colicins [19], [20]. Maximum discrimination of strains was achieved by the combination of serological and biochemical typing [10], [21], [1], [4], [17].

2. MATERIAL & METHODS

Source of Microorganisms:

In this study one hundred-and four (104) Klebsiella strains were collected. They were selected from 1007 hospitalized patients at Sohag University Hospital, The Klebsiella Isolates were demonstrated in 55 (16.3%) isolates of sputum, 34 (7%) isolates of urine and 15 (8%) isolates of septic wound specimens. The samples were handled to the laboratory under strict aseptic conditions without delay otherwise cooled until examined.

Isolation & biochemical identification of Klebsiella spp. [22]

Often the testing requires a Gram stain of the isolate be performed-after motility test- to verify the microscopic morphology before any rapid testing is performed. Two important criteria are required for accurate identification of isolates from normally sterile body sites.

All of the isolates were identified depending on the microscopical, morphological examination, culture characteristics and conventional biochemical reaction tests as indole and methyl red negative tests. Lactose fermenter, growth on citrate as a single carbon source and the Voges- Proskauer test showed positive results. Also urease, catalase, malonate utilization, phenyl deaminase and (arginine, lysine and ornithine decarboxylase tests).

Antibiotic susceptibility test by disc diffusion method

All Klebsiella isolates were subjected to susceptibility testing [23] disk diffusion method on Mueller-Hinton agar plates and the tube serial dilution method- as recommended by the [24] Zone diameters measured and interpreted as resistant, intermediate susceptible or susceptible.

Fourteen types of antibiotics discs containing the following antibiotics (µg /disk) were used:

Ampicillin (AM 10µg), Amikacin (AN 30µg), Aztreonam (ATM 30µg), Chloramphenicol (C 30µg), Clprofloxacin (CIP 5µg), Cefoperazone (CFP 75µg), Cefotaxime (CTX 30µg), Doxycycline (D 30µg). Gentamicin (GM 10µg), Imipenem (IPM 10µg), Noroxin (NOR 10µg), Trimethoprim (TMP 2.5µg), Tobramycin (TOB 10 µg) and Pipracillin (Pip 100 µg).

Serotyping of Klebsiella spp. using slide agglutination test [25]***:***

The specific polyvalent antiserum used, was commercially provided by Biodesign Company, (USA). It was described as rabbit anti-Klebsiella genus specific. It's genus specific protein from *K. pneumoniae* (CAT. No. 847901R). These antisera react primarily with *K. pneumoniae* and cross-react with other *Klebsiella* spp. as well. Monoclonal anti-Klebsiella. (CAT. No. C61206M), (containing pools of 38 K-types), IgG2a was the immunoglobulin type and the antibody reacts with a protein determinant in the cell wall of 38 out of known 77 serotypes Klebsiella but does not react with several *E. coli*, *P. vulgaris* and *M. morganii*. **Method:** Suspensions of the tested bacteria in a physiological salt solution were prepared. Place 10 µl of this solution on a glass slide. Add 10 µl antiserum (polyvalent & monoclonal anti-Klebsiella solution Biodesign company (USA), mix well and watch possible clumping (under microscope). Negative and positive controls were used. (Using undiluted antibody).

Detection of bacteriocin sensitivity and bacteriocin typing by (Scrape &streak method [25]***)***

Eight bacteriocin producer standard strains of Klebsiella K1, K17, K23, K24, K40, K96, K168 ATCC 35599, and K207. Also three indicator strains: K437, K468 and K487 were kindly provided by Prof. Dr. Adolf Bauernfiend (Microbiological Consulting Education Research Institute, MÜnchen, Germany). **Method:** The Klebsiella isolates were tested against (8) bacteriocin producers streaked across the surface of MacConkey's agar plates. After incubation at 37ºC for (18-24) hrs the growth was removed to one side of the plate by means of a microscope slide. The plate was exposed to chloroform vapor for (15 minutes) to kill any living bacteria. After chloroform evaporation (20 minutes), 3 hrs tryptone water-cultures of tested and indicator strains were subsequently streaked across these plates at right angles to the lines of growth of the producer strains. After overnight incubation at 37°C. The plates were examined and any inhibition of growth was recorded. According to [26]. The resulted types according to bacteriocin sensitivity pattern were recorded in the following way as shown in table (1).

Table (1): Demonstration of 45 combinations of inhibition by klebocin producer strains detected by Bauernfiend scale

{1}	**{1,2}**	**{1,2,3}**	**{1,2,3,4}**	**{1,2,3,5,8}**	**{1,2,3,4,5,6}**	**{1,2,3,4,5,6,7}**
{2}	**{1,3}**	**{1,2,7}**	**{1,2,3,7}**	**{1,3,4,5,6}**	**{1,3,4,5,6,7}**	**{1,2,3,4,5,6,8}**
{3}	**{1,7}**	**{1,3,5}**	**{1,3,4,5}**	**{1,3,4,5,8}**	**{1,3,4,5,6,8}**	**{1,3,4,5,6,7,8}**
{4}	**{1,8}**	**{1,3,6}**	**{1,3,4,6}**	**{2,3,4,5,6}**		**{1,2,3,4,5,6,7,8}**
{5}	**{2,3}**	**{1,3,8}**	**{3,4,5,6}**	**{3,4,5,6,8}**		
{6}	**{2,8}**	**{1,6,8}**		**{3,4,5,7,8}**		
{7}	**{3,6}**	**{2,3,5}**				
{8}	**{4,5}**	**{4,5,6}**				
	{4,6}	**{4,6,8}**				
	{6,8}					

- *Each producer strains PI through P8, selected for the typing set shows a characteristic pattern of inhibition that indicated the type between brackets.*
- *Serial K -types of producer strains PI through P8 are: KI. KI7, K23. K24, K40, K96, KI68 ATCC 35599, K207.*

3. RESULTS

Antibiotic susceptibility test of Klebsiella isolates by disc diffusion method:

A high prevalence of multiresistant strains of Klebsiella was observed in this study. Different isolates collected from different specimens revealed different susceptibility patterns to antibiotics. All isolates exhibited multiresistant patterns ranging from 4-12 antibiotics. The results recorded in fig (1) showed that the most of the isolates were resistant to multiple antibiotics including ampicillin 99(95.19%), pipracillin84(80.76%)trimethoprim88(84.61%), chloramphenicol 76(73.07%), doxycycline 73(70.19%), tobramycin57(54.80%)ciprofloxacin 56(53.84%), cefoperazone 51(49.03%), aztreonam42(40.38%),cefotaxime

34(32.69%), gentamicin 40(38.46%), noroxin34(32.69%), amikacin 20(19.23%), and imipenem 5(4.80%).

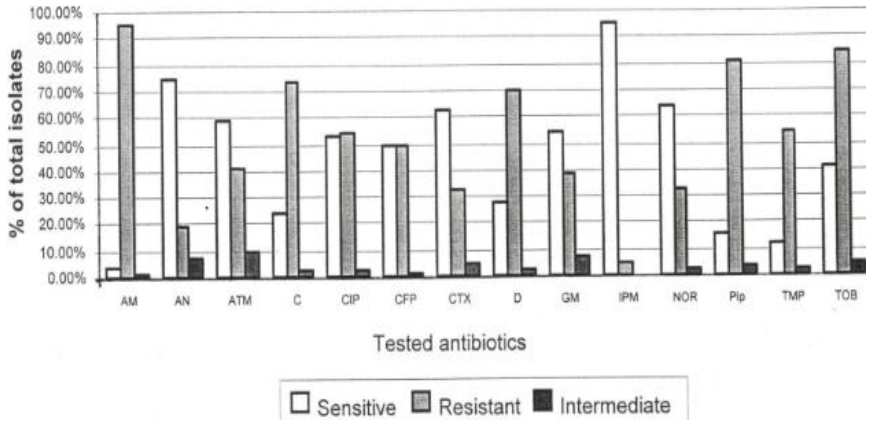

Fig.(1):Sensitivity pattern of total Klebsiella isolates

Serological identification of genus Klebsiella

All isolates of Klebsiella (104 strains) were tested with the genus-specific polyvalent antisera by using slide agglutination test.

Serotyping of Klebsiella species

99 (95.1 %) out of (104) tested strains of Klebsiella isolates shown positive agglutination reaction with polyvalent antibody (specific to 38 K-types) resembling *K. pneumoniae* serotype.

Bacteriocin sensitivity of isolated strains of Klebsiella

The results of bacteriocin sensitivity of the isolated strains of Klebsiella (104) was estimated by the inhibition pattern they form against 8 reference bacteriocin producer strains as shown in table (2) and fig (2,3). Such sensitivity was recorded in this finding from which it was observed that most of the isolated strains of Klebsiella were sensitive to more than one of the producer strains 87 (83.6%). However 17 (16.3%) strains were found to be sensitive only to one of these producers. {P1}& {P8} bacteriocin producer strains have the broad spectrum of activity, where they inhibited 74 (71.15%) & 70 (67.30%) of the 104 tested strains respectively. Sputum isolates were more sensitive to {P1} & {P8} than pus isolates. While both bacteriocin producers {P2} & {P7} have the narrowest activity 11(10.57%) of the total isolates. The least sensitive strains to {P2} & {P7} were pus isolates, where 2 (13.33%) and 0.0 (0.00%) were sensitive to these strains respectively.

Table (2): Sensitivity of total Klebsiella isolates to reference bacteriocin producing strains

Specimen / Bacteriocin Producers	Total (104)	
	No.	%
P1	(74)	71.15%
P2	(11)	10.57%
P3	(30)	28.84%
P4	(15)	14.42%
P5	(20)	19.23%
P6	(20)	19.23%
P7	(11)	10.57%
P8	(70)	67.30%

Bacteriocin (Klebocin) typing of Klebsiella strains

Twenty-one different combinations of bacteriocin sensitivity patterns were observed. There were some differences in patterns between clinical isolates (pus, urine, and sputum).Bacteriocin types {1,8}, {1,3,8}, {1} and {1,6.8} were the most prevalent bacteriocin types in isolated strains of Klebsiella, where they recorded in 29 (27.88%), 14 (13.46%), 9 (8.65%) and 8 (7.69%) of the total isolates respectively. Whereas bacteriocin types{2,3,5},{3,4,5,7,8},{1,3,4,5,6,7}and {1,2,3,4,5,6,7,8} were the least prevalent types (one strain of each type). Other bacteriocin types were recorded in somewhat variable percentages as shown from fig. (4). The most predominant bacteriocin types in sputum and urinary isolates are {1,8},{1,3,8},and,(1,3,8} and in wound sepsis isolates are {6,8} and {1,8}. Two (3.6%) of each bacteriocin types {1,7}, {1,2,3,7}, and 1(1.8%) of types {3,4,5,7,8} & {1,2,3.4,5,6,7,8} were observed only among respiratory isolates.

Fig. (2,3): Inhibition pattern formed by P1 reference klebocin producing strains

Klebocin type {1,8} represented by strains no.(3,7,13) and indicator strain I_3 Bacteriocin types

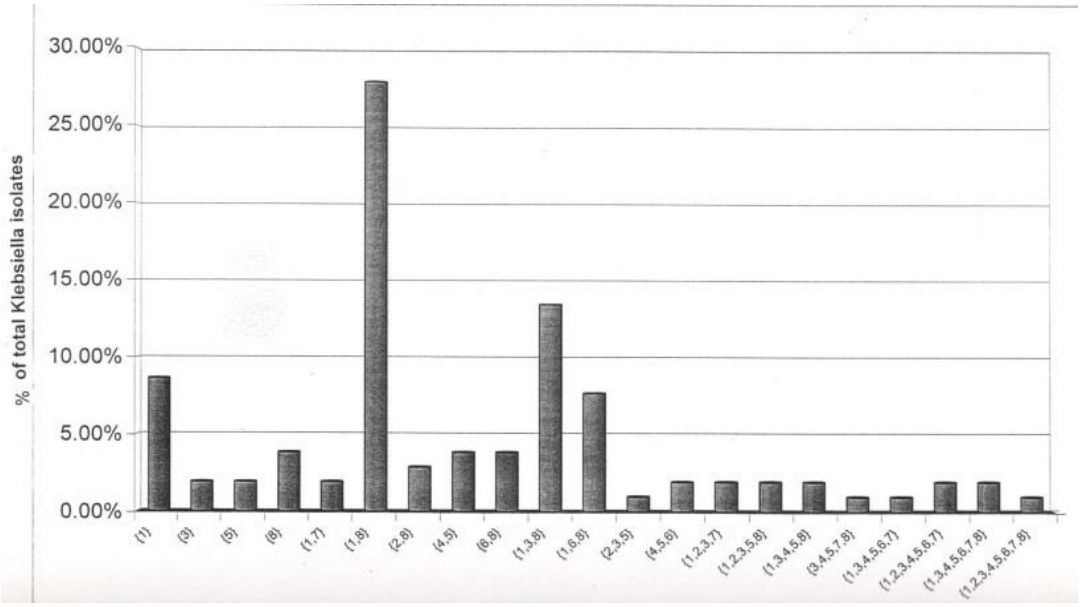

Fig. (4): Klebocin typing among total Klebsiella isolates according to combination of inhibition pattern

4. DISCUSSION

Nosocomial infections of multiresistant *K. pneumoniae* have a significant impact on clinical practice, morbidity, mortality and costs. Spread of these organisms within the hospital via person-to- person contact or from environmental reservoirs is an important factor in nosocomial infection. Thus, identification of the source of infection and the route of transmission is essential to allow appropriate nosocomial infection control [(27), (2)]. In this study the Klebsiella isolates were demonstrated in 55 (16.3%), 34 (7%) and 15 (8%) of respiratory, urinary and wound sepsis respectively. They were represented 104 (10.32%) of total nosocomial bacterial infections. This is in agreement [(2)] being the primary cause of respiratory tract infections like pneumonia, rhinoscleroma, ozaena, sinusitis and otitis media. Biochemical identification by using conventional methods[(22)]. The isolated strains showed similarity in their biochemical reactions.

Sensitivity of isolated Klebsiella to (14) antibiotics by disc diffusion method

In this study a high prevalence of multiresistant Klebsiella isolates were observed. Different isolates collected from different specimens revealed different susceptibility patterns to antibiotics. All isolates exhibited multiresistance patterns ranging from resistance to 4-12 drugs. This coincides with [(28), (29)] who found that the level and spectra of resistance in the pathogens were the same with respect to 17 drugs also [(28), (30)] who, found that all (100%) Klebsiella today are resistant to ampicillin because of a common B-Iactamase.

Serotyping of genus Klebsiella

Capsular serotypes K1 and K2 are considered as predominant virulent strains of *K. pneumonia* [(31), (32)] Agglutination was carried out on glass slides [(8)], Klebsiella are generally typed by the method of capsular serotyping but, although this is a reliable method, it is time consuming, requires the production of a large number of antisera and is not easily available [(33)]. Quellung technique was limited, because of costs of various antisera preparation [(34)]. According to recent epidemiologic studies, 80% of cases were caused by *K. pneumoniae*; 60% to 80% of the *K. pneumoniae* isolates causing these cases belonged to the K1 capsular type, and 10% to 14% isolates belonged to the K2 capsular type in Asia [(35)] Monoclonal antibodies (MASs) or antisera specific for the O-polysaccharide antigen D-galactan II, O- galactan II or core oligosaccharide of the Klebsiella O1 antigen [(36)]. All the isolated strains of Klebsiella in this investigation, (104) were confirmed to be Klebsiella by testing against genus-specific polyvalent antisera by using slide agglutination test, where [(37)] reported that 89.4% of Klebsiella isolates reacted with the genus-specific monoclonal antibodies, which recognizes an epitope of the outer core region of Klebsiella lipopolysaccharide. KP-1 reacted to 52% *K. pneumoniae* clinical isolates [(38)], whereas KP-2 reacted to 62% of these isolates. Standard *K. pneumoniae* was positive to all the three antibodies. In this investigation 99 (95.1%) out of 104 tested strains showed positive agglutination reaction with polyvalent antibody

(containing pools of 38 K-types) resembling *K. pneumoniae* serotype. However, [33] found that among 77 reference strains for Klebsiella K-types there were 53 (68.8%) strains of *K. pneumoniae*. The capsular types of these isolates were determined by the gel precipitation reaction. Whereas [38] found that, hyperimmune sera (polyclonal) to CPS detected 76% *K. pneumoniae* clinical isolates as positive.

Sensitivity of Klebsiella strains to reference bacteriocin producer strains

Bacteriocins are ribosomally synthesized peptides that have bacteriostatic or bactericidal effects on other bacteria [18], [39]. The action of a bacteriocin is restricted to only the limited number of related species, and some act only on certain strains of the same species, which produce them. The action of bacteriocins is conditioned by the presence of specific receptors [40].The mechanism of lethal action of " killer "strain is carried out either directly or indirectly against certain functions of the target cell ([41], [42], death of the sensitive strain is produced as a consequence of transport collapse at membrane level resulting in a drastic of indispensable metabolites or ions in other cases, the antibacterial effect produces blocking of macromolecule synthesis or loss of metabolite stabilization within the cell altering the basic metabolism. [41]. Determination of the biochemical, bacteriocin (susceptibility to 8 bacteriocins), phage types, and MIC for 9 antibiotics. Basing on these methods it was found that 41 types of *K. pneumoniae*, 13 types of *K. oxytoca* and one type of *K. ozenae occur* [43], [44], [21]. Consequently, in the present work of bacteriocin sensitivity by using a scrap & streak method. It was observed that most of the isolated strains of Klebsiella were sensitive to more than one of the klebocin producer strains [87 (83.6%) of 104 tested strains]. However 17 (16.3%) isolates were found to be sensitive to only one type of these producer strains. This is in agreement with the finding of [26] who concluded that (91%) non epidemic clinical isolates of *K. pneumoniae* were inhibited by one or more of 8 producers. In contrast to these results [45] concluded that among set of 10 Klebocin-producing, only one of which predominated due to the sensitivity of the Klebsiella to two klebocin L4, and D33 and is presumably associated with their relatively low titers. The obtained results concerning sensitivity of Klebsiella isolates as in tables 19, 20, 21 and 22 show that {1} & {8} bacteriocin producers had a comparatively broad spectrum of activity, where they inhibited 74 (71.15%) & 70 (67.30%) of the total strains respectively. Also, 30 (28.8%) of all isolates were susceptible to bacteriocin type 3.

This finding is similar to that of [46], [47] where they found that the *K. pneumoniae* strains showed a certain bacteriocins showed a very broad spectrum of activity; e.g. (93%) of all isolates were susceptible to bacteriocin type 3. Also, [48] found that bacteriocin types 1 and 3 showed a very broad spectrum, more than (95%) of the isolates being sensitive to one of these bacteriocins.

Klebocin typing of Klebsiella strains

Bacteria may be typed by their sensitivity to bacteriocins produced by reference strains. [26] Bauernfeind, et al. Klebsiella marker systems include determination of susceptibility patterns, serotype, bacteriocin susceptibility, bacteriophage susceptibility, biotype, and plasmid content, size and endonuclease fragment size. Bacteriocin susceptibility typing is easier and cheaper but reproducibility is sometimes poor depending on the producer strains used. No one method is ideal, and characterization of isolates from an outbreak done by using several different marker systems, [43], [44].The combination of antibiogram and klebocin typing methods possess a significant contribution in epidemiological studies. [49]. It is evident from tables 13, 14, 15 and 16 that there were 21 different combinations of bacteriocin sensitivity patterns were observed. This finding is similar to [50] who noticed that bacteriocin typing differentiated *K. pneumoniae* to 20 klebocin types. In addition, to [48] concluded that 21 different patterns were observed. Type {1}, {3,4,5,6}, {8} was most common in both species (*K. pneumoniae, K. oxytoca*). Our findings are nearly similar with the results of [45] who used a set of 10 Klebocin-producing strains for typing 630 clinical isolates of Klebsiella. They found that most strains belonged to 16 bacteriocin types; only one type of them was predominated. Out of 104 *K. pneumoniae* in this investigation 97 (93.3%) were bacteriocin typed using a set of 8 producer strains. These results are in agreement with those of [46] where they found that 96% out of 452 *K. pneumoniae* and *K. oxytoca* strains could be bacteriocin typed using the same set of 8 producer strains. Also, they were in agreement with finding of [50] who observed that bacteriocin typing differentiated 70 (84.3%) out of the 83 isolates. Many investigators reported that bacteriocin typing could differentiate a high percentage of Klebsiella isolates ranged from 67% -96%. [43], [44]. On other hand and by using a set of 6 standard bacteriocin-producer strains. In this investigation the most predominant bacteriocin types were {1,8} represented by 29 (27.8%) and type {1,3,8}, represented by 14 (13A%) of the total isolates. Chhibber et al. [51] used 6 standard klebocin-producer strains (153-158). They found that the most predominant klebocin types were 244 (14.3 %), 313 (13.7%) and 113 (7.6%). Klebocin types 314 and 111 each contributed 5.2%. In this study the most predominant klebocin types in respiratory isolates were

{1,8}, and {1,3,8}, in urinary isolates were {1,8}, {1,6,8} and in wound sepsis isolates were {1,8}, and {6,8}. Bacteriocin type {2,3,5} appeared only among pus isolates. In contrast [51] showed that no significant correlation was observed between the source of isolation and the klebocin type.

5. CONCLUSION

Spreading, of hospital infections of multidrug- resistant *Klebsiella* spp. are often caused by a new type of strain, the ESBL producers. These findings confirm the alarmingly high rates of multiresistance and the emergence of ESBL-producing strains. Typing of multiresistant strains isolated from patients, staff, medical devices and the environment can assist in this process. The combination of phenotypic markers such as biotyping and serotyping and bacteriocin typing was more discriminating than either method alone to provide microbiologists and physicians with critical information for managing and controlling the intra and inter hospital dissemination of resistant pathogens and also for epidemiological studies.

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