Antimicrobial spectrum of the action of bacteriocins from *Klebsiella* isolates from Hilla/Iraq

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ABSTRACT:

Background and objectives: Bacteriocins are antibacterial proteins produced by bacteria**.** They also differ from traditional antibiotics in having a relatively narrow spectrum of action and being lethal only for bacteria which are closely related to the producing strains**.** The aim of this study was to investigate the antibacterial spectrum of the action of bacteriocins from environmental and clinical *Klebsiella pneumonia*e isolates from Hilla/Iraq.

Materials and Methods: The antibacterial activity of bacteriocins (klebocins) from *Klebsiella pneumoniae* isolates against different pathogenic species of Gram-negative and Gram-positive bacteria (by cup assay method) was determined.

Results and Conclusion: The results revealed that brain heart infusion agar medium supplemented with 5 % glycerol was the best culture medium used for detection of klebocin-producing isolates and cup assay was the best method used for such purpose. Results also revealed that klebocins of *Klebsiella* isolates had a broad antimicrobial spectrum and active, in addition to *Klebsiella* strains, on many pathogenic species of Gram-negative and some Gram-positive bacteria. Agarose gel electrophoresis of DNA samples of some selected klebocin-producing *Klebsiella* isolates showed that they harbor plasmid bands different in size and position. Results of conjugation experiments revealed that genes encoding for the production of klebocin were located on conjugative plasmids. The heterologus activity of klebocins on many pathogenic species of Gram-negative and some Gram-positive bacteria especially those isolated from patients with chronic pyelonephritis and chronic otitis media, may indicate that these klebocins could be used as alternative to the broad-spectrum antibiotics.

Key words: Bacteriocins, klebocins, antimicrobial spectrum, *Klebsiella pneumoniae.*

INTRODUCTION:

Bacteriocins are antibacterial proteins produced by bacteria. They are found in almost every bacterial species examined to date, and within a species tens or even hundreds of different kinds of bacteriocins are produced. They also differ from traditional antibiotics

in having a relatively narrow spectrum of action and being lethal only for bacteria which are closely related to the producing strains. Many different bacteriocin groups have been described and named after a species or genus of bacteria since 1925 when Andre Gratia discovered the first bacteriocin in *E. coli* (1).

The bacteriocin family includes a diversity of proteins in terms of size, microbial targets, mode of action, and immunity mechanism. The most extensively studied the colicins produced by *E. coli* (2). Bacteriocins (klebocin) of *Klebsiella* have been first described by Hamon, and Peron in 1963 (1)

Epidemiological investigations on *Klebsiella* colonization and disease have relied on *Klebsiella* marker systems, for some part, on bacteriocin (klebocin) typing which based on the sensitivity of *Klebsiella* to bacteriocins produced by set of producers (3, 4, 5, 6, 7).

As a type of bacteriocins, klebocins have a narrow spectrum of action and are lethal only for bacteria which are closely related to the producing strains (homolo-gous activity) (8). On the other hand it was reported that the antimicrobial spectrum of klebocins of *Klebsiella pneumoniae* was broad and was not limited by the frames of the genus and family (9, 10).

MATERIAL AND METHODS:

Isolation and identification of bacterial isolates:

The present study included collection of 298 samples from different environmental and clinical sites during the period from January to July 2003. Environmental samples included: 48 sewage, 10 still water, 6 fountain water, 8 tap water, 9 toilette seat swab, 8 skin ointment. Clinical samples included: 108 urine**,** 50 stool, 6 blood, 27 ear swab**,** 3 wound, 2 burn swab, 4 skin swab, 5 vaginal swab, 4 throat swab. Clinical samples were collected from the main three hospitals in Hilla/Iraq (Teaching hospital, Margan hospital, Maternity and pediatric hospital), in addition to General Health Lab, and some private laboratories in Hilla /Iraq.

Bacterial isolates were identified to the level of subspecies using the traditional biochemical and morphological tests described by (11) and then confirmed using rapid identification systems (API 20 E) as recommended by the manufacturer (Biomérieux/ France).

Detection of Bacteriocin Production :

All environmental and clinical isolates were examined for their ability to produce bacteriocin (klebocin). All the clinical isolates were used as indicator isolates to the klebocin of producer environmental isolates. Cup assay method described by (12) was used for detection of bacteriocin production.

Study of heterologus activity of klebocins:

Cup assay method was also used for detection of the effect of bacteriocin produced by *Klebsiella* isolates on different G-ve and G+ve pathogenic bacterial species (obtained from General health lab, Babylon province/Iraq). One *Pseudomonas aeruginosa* isolate was isolated from a patient with chronic otitis media (COM)**.** Two isolates of *Staphylococcus epidermidis* were isolated from patients with chronic pyelonephritis**.** Standard strain *E.* coli 25922 was obtained from ATCC,USA.

Plasmid analysis

Plasmid DNA was extracted from cultured cell using the alkaline-SDS method described by Pospich and Neumann (13). The bacterial conjugation was carried out using the procedure described by O'Connell (14) in order to detect the role of plasmids in transferring of klebocin production. Klebocin-producing isolates were considered as donor cells against the standard strain *E. coli* J53 (Gift from Dr. George Jacoby, Massachusetts, USA) as a recipient cell. The plasmid profile of the transconjugants, donor cells, and recipient cells was detected using agarose gel electrophoresis. The transconjugants were tested for their ability to produce for their ability to produce bacteriocins.The results were compared with those obtained from original isolates.

RESULTS:

Results of morphological and biochemical characterization tests revealed that a total of 88 isolates were belonged to *Klebsiella pneumoniae*. The influence of growth media and media constituents on bacteriocin production by *Klebsiella pneumoniae* was studied. Several solid and liquid culture media were used for detection of klebocin production by *klebsiella* isolates. Results of (**Table-1**) show that Brain Heart Infusion medium (BHI) supplemented with 5 % glycerol was the best culture medium selected for this purpose.

Results of this study revealed that 55 *klebsiella* isolates (62.5%) of total *klebsiella* isolates (88 isolates) were able to produce klebocin and form a clear and relatively large inhibition zones (10-18 mm) on solid medium. Out of these klebocin-producing isolates, 38 (69.1%) were isolated from environmental samples, and 17 (30.9%) from clinical samples**.** It was found that 82.7% of all clinical *klebsiella* isolates (No.=29) were susceptible to bacteriocin produced by Klebocin-producing isolate E 38, and 75.8% of these isolates were susceptible to bacteriocin produced by isolate E 40.

It was also found that out of 38 klebocin- producing isolates, eight (21%) were able to inhibit growth of more than 50% of the indicator isolates, and 37.9 % of the indicator strains were susceptible to bacteriocins produced by 55% of all producing isolates.

The effect of klebocin produced by *Klebsiella* isolates on some bacterial genera and species was studied. Five klebocin-producing isolates were used for studying activity of klebocin on some pathogenic bacterial isolates: *K. pneumoniae* E 38, E 40, E 51, E 52, and E 54.

Heterologous Activity of Klebocin on Pathogenic Bacteria:

The results of (**Table-2**) revealed that the klebocins from *Klebsiella* isolates were active on several Gram-negative and Gram-positive bacterial species. Most Gram-negative rods were affected by klebocins of *Klebsiella* isolates. *Pseudomonas aeruginosa* isolate was very sensitive to klebocin of *K pneumoniae* E 51giving a large inhibition zone (33 mm). All Klebocin-producing isolates (**Table-2**) had bacteriocinic effects on *Moraxella*

(**Table-1**): Efficacy of different culture media used for detection of klebocin using cup assay method.

Medium	Liquid (BHI + 5% glycerol) Solid (BHI +5% glycerol)	Liquid (TSB) Solid (TSA)	Liquid (cooked meat broth) Solid (TSA)	Liquid (Different types) Solid (NA or BHI agar)
Efficacy	+++ clear and large inhibition zones (10-18 mm)	+ unclear and small inhibition zones (5-10 mm)	+ unclear and small inhibition zones (5-10 mm)	– No klebocin-producers can be detected

BHI, Brain heart infusion; TSB, Tryptic soy broth; TSA, tryptic soy agar; N.A., Nutrient agar. – No production; + weak production; +++ very good production of klebocin.

catarralis com1 and com2. The latter species was very sensitive to klebocin of *K. pneumoniae* E 51, giving a large inhibition zone (23 mm).

Results of (**Table-2**) also showed that all Gram- positive bacteria were resistant to the tested klebocins, except *Staphylococcus aureus* isolate, which was sensitive to klebocins of *K. pneumoniae* E 38 and E 51, as well as *Staphylococcus epidermidis* cpn1 isolated from patient with chronic pyelonephritis, which was sensitive to klebocin of *K. pneumoniae* E 54 only.

Table-2:Activity of klebocins of *K. pneumoniae* isolates on some pathogenic bacteria.

Test organism	Activity (+)* of klebocin- producing isolate				
	E 38	**E 40**	**E 51**	**E 52**	**E 54**
E. coli ATCC 25922	+	+	+	+	+
E. coli EC1	-	-	+	+	-
E. coli EC2	+	+	-	+	-
Enterobacter aerogenes	+	+	+	+	+
Proteus mirabilis SM1	+	+	+	-	-
Proteus mirabilis SM2	+	+	+	+	+
Pseudomonas aeruginosa	+ (20)	+	+ (33)	+	+
*Moraxella catarralis*com1	+	+	+ (17)	+	+
*Moraxella catarralis*com2	+	+	+ (23)	+	-
Staphylococcus aureus 20	+	-	+	-	-
Staphylococcus aureus 21	-	-	-	-	-
Staph. epidermidis cpn1	-	-	-	-	+
Staph. epidermidis cpn2	-	-	-	-	-
Enterococcus faecalis 11	-	-	-	-	-
Enterococcus faecalis 12	-	-	-	-	-

+ refers inhibition zones ranged from 10-15mm, except otherwise indicated (between brackets).

The electrophoresis results of klebocin-producing isolates (**Figure-1**) showed that the isolates were different in their plasmid profiles and the plasmids were different in size. The results showed that the isolate *K. pneumoniae* E 38 (Lane D), harbors a single mega plasmid band , and *K. pneumoniae* E 40 (Lane E), possesses one small plasmid.

Results of (**Figure-2**) revealed that the conjugation between the standard strain *E. coli* J 53 and each of the *Klebsiella* isolates was successful. The conjugation frequency for these transconjugants was relatively low (2×10^{-6}). The result obtained with klebocin-producing isolate E 38 which possesses one large plasmid (Lane D) showed that this mega plasmid was transferred to the recipient cell (lane E) during. This result indicates that this large plasmid was a conjugative plasmid conferring klebocin production to the recipient cell.

The transconjugants resulted from conjugation between klebocin-producing isolates and the standard *E. coli* J53, were detected for their ability to produce klebocin using cup assay method. Results showed that the transconjugants were able to produce klebocin on tryptic soy agar and inhibited the growth of some pathogenic Gram-negative enteric rods, but they were unable to inhibit the growth of all other bacterial species tested. The klebocin inhibition patterns of the transconjugants were resembled to that formed by the original donor cells.
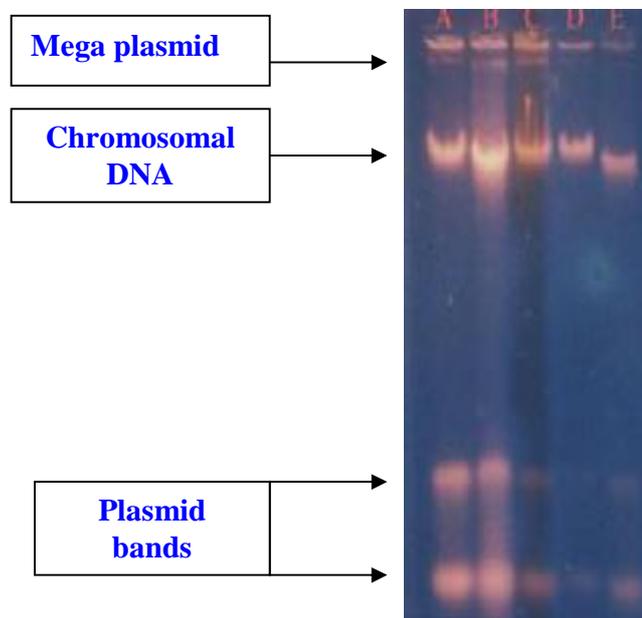

Figure 1. Agarose gel electrophoresis of plasmid profiles of *K. pneumoniae* strains isolated from clinical and environmental samples: Lanes: A, 6 C; B, E 54; C, 22 C; D, E 38; E, E 40.
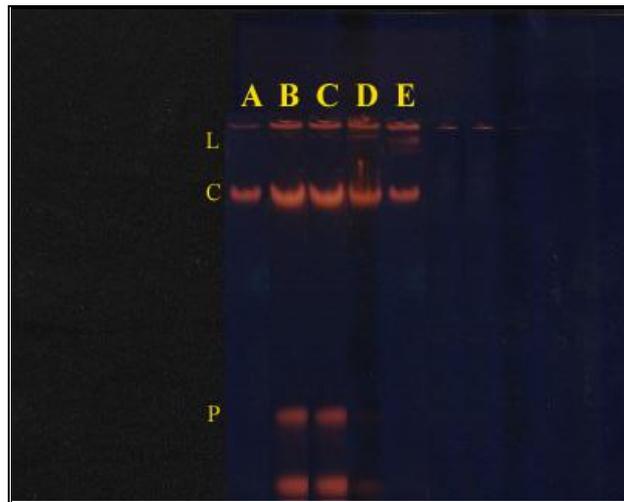
Figure 2. Agarose gel electrophoresis of plasmid DNA from wild type isolates of *K. pneumoniae* and their transconjugants (in *E. coli* J53). Lanes: A, standard strain J53; B, 6 C; C, transconjugant resulting from conjugation between *K. pneumoniae* 6C with standard strain *E. coli* J53, D, E 38 isolate; E, transconjugant resulting from conjugation between *K. pneumoniae* E 38 with standard strain J53.

DISCUSSION:

The influence of growth media and media constituents on bacteriocin production by *Klebsiella pneumoniae* was studied by several authors (15, 16, 17). Vignolo *et al.*, (16) found that maximal bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors, such as sugars, vitamins and nitrogen sources, by regulating pH or by choosing the best-adapted culture medium. In this study, Brain Heart Infusion medium (BHI) supplemented with 5 % glycerol was the best culture medium selected for this purpose and cup assay method was the best method used for detection of bacteriocin production. These findings are in agreement with the results obtained by many researchers (12, 18) who found that cup assay method was the best method used for detection of bacteriocin-producers lactobacilli and *E. coli* strains, respectively.

Solid medium is better than liquid medium for detection of bacteriocin production, since the producing isolates can be distinguished simply by inhibiting sensitive isolates and because bacteriocin is induced in the presence of indicator bacterial cells (that have the binding receptors), which are absent in the liquid medium. While in the solid medium, the presence of these bacterial cells will encourage the producing strains to produce bacteriocin (19).

Results also revealed that the percentage of klebocin-producers was close to that reported by (5), who found that 63% of *Klebsiella* strains were found to be bacteriocin-producers, and much more than that reported by other studies (4, 20).

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Many researchers have used standard klebocin producers (8 strains) for detection of klebocin production and typing of *klebsiella* isolates. These isolates were typed by their susceptibility to bacteriocin synthesized by producer standard strains (3, 6, 7). However, production of bacteriocin by *klebsiella* spp. can be detected using *Klebsiella* isolates sensitive to k*lebsiella* bacteriocin as indicators. In the present study, klebocin production by *K. pneumoniae* isolates was detected using local clinical isolates as indicators without employing standard producer strains because these local isolates were sensitive to the klebocin produced by the environmental isolates.

It was found that bacteriocin activity of *E. coli* isolates is not essential for virulence and pathogenicity of the producing isolates, but it aids them in their competition (21).

In another study, the ability of bacteriocin production in *E. coli* strains, isolated from urine of patients suffering of UTI and from stool of healthy individuals, was tested. It was found that there was no significant difference in ability of these strains to produce bacteriocin, between those isolated from urine or stool samples, which indicates that the bacteriocin is not a virulence factor (22). It was also found that in a mixed fermentation environment, production of bacteriocins may prove advantageous for a producer organism to dominate the microbial population (23).

Broad spectrum of activity of klebocin produced by *klebsiella* strains was reported by other studies. Podschun and Ullmann (7) revealed that certain bacteriocins showed a very broad spectrum of activity; and 93% of all *klebsiella* isolates were susceptible to bacteriocin type 3 produced by one of their standard Klebocin-producing isolates. They also evaluated whether clinical *Klebsiella* isolates differ from nonclinical strains with respect to bacteriocin susceptibility patterns, and their results suggested that nonclinical *Klebsiella* strains did not show other bacteriocin susceptibility types than clinical isolates do.

The results of (**Table**-**2)** revealed that the klebocins from *Klebsiella* isolates were active on several Gram-negative and Gram-positive bacterial species. These results are in agreement with findings obtained by several authors who reported that the antimicrobial spectrum of the bacteriocins from *Klebsiella* strains studied was broad and was not limited by the frames of the genus and family (9). It was found that bacteriocins from *Klebsiella* strains were active against *Klebsiella, Enterobacter, Escherichia, Shigella*, *Proteus* and *Pseudomonas*. These bacteriocins were also capable to protect corn and tomato seeds from contamination with *Erwinia* (6, 10).

Albesa *et al.*, (9) reported that two of 36 *K. pneumoniae* strains had bacteriocinic effects on homologous species and they also acted on coagulase positive and negative staphylococci which indicated that homologous activity on *K. pneumoniae* seemed to be undistinguishable from the compound with heterologous action on staphylococci in the aspects that were characterized in their work.

Results of another study found that bacteriocins from *K. pneumoniae, K. ozaenae,* and *K. rhinoscleromatis* were active on *Klebsiella, Enterobacter, Escherichia, Shigella,*

and *Proteus* whereas all cultures of *Agrobacterium , Corynebacterium , Micrococcus , Staphylococcus,* and *Streptococcus* were resistant to the action of these klebocins (10).

Sensitivity of the pathogenic bacterial isolates to klebocins, especially those isolated from patients with chronic pyelonephritis and chronic otitis media, may indicate that these klebocins could be used as alternative to the broad-spectrum antibiotics. In this respect, the klebocins are considered as a "designer drugs" which target specific bacterial pathogens and subsequently each antibiotic is used infrequently, which result in a reduction in the intensity of selection for bacterial resistance (4).

The difference in plasmid profiles and in size of plasmids of *Klebsiella pneumoniae* isolates in the present study was in agreement with that reported by Podschun *et al.*, (25) who showed that plasmids of *K. pneumoniae* isolated from human patients were distributed widely and showed great diversity. The possession of *K. pneumoniae* E38 isolate a single mega plasmid band (**Figure**-**1**) is in agreement with findings obtained by Al-Barzangi (24) who showed that all her bacteriocin producer strains of *Enterococcus faecalis* harbor a single mega plasmid encoding bacteriocin production.

It can be concluded from the results of conjugation experiment (**Figure**-**2**) that the expression of klebocin production, was plasmid-encoded, transferred to the standard strain, which received these properties. Because of their transfer among bacterial genera as well as their facilitating transfer of non-conjugative plasmids, the conjugative plasmids are considered very dangerous, being able to confer resistance to a large numbers of antibiotics in addition to production of klebocin..Gene transfer may occur across a very broad host range, such as between Gram-negative and Gram-positive bacteria (26).

The klebocin inhibition patterns of the transconjugants (resulted from conjugation between klebocin-producing isolates and the standard *E. coli* J53) were resembled to that formed by the original donor cells. The acquisition of ability to produce klebocin by these transconjugants, may refer to that this trait was present on mega plasmid (in *K. pneumoniae* E 38) that transferred to the host cell during conjugation. However, it's not applicable to *K. pneumoniae* E40 which harbors one small plasmid. The only possible interpretation of successful conjugation in this isolate, is that it may harbors another large conjugative plasmid, responsible for klebocin production, which was not appeared during electrophoresis. This result is in agreement with that reported by Al-Barazangi (24) who showed that bacteriocin producer isolate of *Enterococcus faecalis* harbor a single mega plasmid band and the genes encoding bacteriocin production and cephalexin resistance were located on a conjugative plasmid which was refractory to the curing agents used. However, this results disagree with findings obtained by Chhibber and his coworkers who reported that conjugal intrageneric transfers, elimination experiments with various curing agents, and plasmid isolation procedures showed that *Klebsiella pneumoniae* strain 5 did not harbor any plasmid. Hence chromosomal location of the genetic determinants was suggested (27).

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