Antibacterial and antibiofilm activity of klebicin crude extract on clinical isolates of *Salmonella* and *Enterobacter*

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Abstract. To date, studies on the effects of klebicin on biofilm development are limited. The aim of the present study was to determine the effect of klebicin on biofilm formation, as it is an important factor in the pathogenicity of bacteria. Klebicins (bacteriocins), which are naturally occurring antimicrobial peptides synthesized by ribosomes, were produced by *Klebsiella* species. The action of klebicin is typically focused on bacteria, which are closely associated to the strains that produce it. This investigation intends to identify specific klebicin genes in isolated *Klebsiella pneumoniae* (*K. pneumonia*). Biochemical testing and the VITEK-2 compact system were used to identify and confirm 32 *K. pneumonia* isolates. Susceptibility testing was performed for six antibiotics (gentamycin, ciprofloxacin, azithromycin, tetracycline, cefotaxime and chloramphenicol) against *Salmonella* and *Enterobacter*. The results revealed that the majority of both bacterial isolates were resistant to the antibiotics. DNA was extracted from 32 *K. pneumonia* isolates and the results revealed that the klebicin gene was detected in 31 (96.87%) of the isolates. Klebicin-like substance was extracted, and the minimal inhibitory concentration of klebicin extract was assessed against *Salmonella* and *Enterobacter* isolates. The results revealed that this concentration ranged between 25-50% for *Salmonella* and 50% for *Enterobacter*. The antibiofilm effect was assessed against the *Salmonella* and *Enterobacter* isolates, as well as against the dual biofilm formation of both bacteria. It was found that the reduction of biofilm in the dual bacterial isolates was greater than that of each type of bacteria alone. On the whole, the findings of the present study data suggest the possibility of using bacteriocin (klebicin) as an antibacterial and antibiofilm agent.

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Introduction

The most frequent growth pattern used by bacteria, the biofilm, is now understood to have significant therapeutic implications (1). Bacteria which are in groups and that have created a self-made matrix and are attached to a surface, as well as to one another are known as bacterial biofilms. The biofilm matrix is composed of proteins (such as fibrin), polysaccharides (such as alginate) and extracellular DNA. Biofilms formed by bacteria can use a range of ways of survival mechanisms that enable them to bypass the host's defensive mechanisms in addition to the refuge provided by the matrix (2,3). Treatment is made more difficult by the development of bacterial biofilms as the illness progresses. Enclosed cells inside the biofilm have distinct traits that lead to an increase in antibiotic resistance, which is greater to that of the planktonic condition, by 10 to 1,000-fold (4).

Antibiotic resistance is known as a serious health issue and is mostly caused by the improper and excessive usage of antibacterial substances (5). Humans naturally acquire typhoidal and non-typhoidal *Salmonella* from the environment or diet. *Salmonella* can cause diarrheal disease, and can also create biofilms in the intestines after being consumed by the host; *Salmonella* Typhi can move into the gallbladder and develop biofilms on cholesterol gallstones after obtaining access to the liver systemically. These biofilms in humans enable both chronic *Salmonella* infection and ongoing *Salmonella* host shedding (6).

Antimicrobial proteins (AMPs), plant-derived antimicrobial compounds, probiotics and bacteriophages as biological alternatives to antibiotics are increasingly being employed for the prevention and treatment of bacterial pathogenic illnesses (7). Gram-negative bacteria, including Escherichia *coli* (*E. coli*), *Klebsiella* and *Pseudomonas* spp. produce AMPs known as bacteriocins, which have narrow-spectrum action against Gram-negative infections. Colicins, colicin-like bacteriocins, microcins and phage tail-like bacteriocins are the four categories into which they fall. Gram-negative bacteria that make bacteriocins comparable to *E. coli* colicins in structure and function include *P. aeruginosa* (e.g., S-type pyocins) and *Klebsiella* spp. (8,9). Numerous types of bacteria create bacteriocins, which are water-soluble protein toxins, in response to nutritional deprivation and intra- and interspecies competition (10). The bacteriocins produced by *Klebsiella* are known as klebocins or klebicins (11). Klebocin is released

from *Klebsiella pneumoniae* (*K. pneumoniae*), and it appears to exert effects against a variety of bacteria belonging to the Enterobacteriaceae family (12). *Salmonella*, *Pseudomonas* and *E. coli* are examples of Gram-negative organisms that can be controlled by bacteriocins, a class of non-antibiotic antibacterial proteins (9). Klebocins are harmful for *Klebsiella* species with a klebocinogenic plasmid that carries the genetic components for klebocin synthesis, immunity and release. Additionally, it was discovered that klebocins are chromosomally encoded. The genetic analysis of the antibiotic system of klebocin has demonstrated that it comprises proteins, since it is expressed by distinct regulatory genes (13). The specific class of bacteriocins known as klebocins exclusively exhibits homologous action, or activity against bacteria that are closely related to one another (14). By contrast, it has been indicated that the antibacterial spectrum of klebocins from *K. pneumoniae* is broad and unrestricted by the confines of the genus or family (15). Thus, the present study aimed to detect klebicin in *K. pneumoniae* isolates and to evaluate their antimicrobial activity on biofilm formation by other pathogenic bacteria, such as *Salmonella* and *Enterobacter.*

Materials and methods

Isolation and identification of K. pneumonia and Enterobacter. The protocol for the present study was approved by the Ethics Committee at the Department of Biology, University of Baghdad and the Iraqi Ministry of Health (Reference: CSEC/0323/0056). Written informed consent was obtained from all the patients. The study was carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki). Different wound samples (surgical wounds and burn wounds; n=120; 67 males and 53 females) Wound samples (n=120) were collected from Baghdad hospitals in Iraq (Al-Yarmouk Teaching Hospital and Baghdad Teaching Hospital). Bacterial isolates were identified using the traditional biochemical and morphological tests. They were cultured on MacConkey agar (HiMedia Laboratories Private Limited), and examined morphologically bacterial shape, size and arrangement using an optical microscope. (Olympus Corporation) A motility test was performed by stabbing in a semisolid medium, and the suspected isolates were confirmed using the VITEK® 2 Compact system (bioMérieux France).

Isolation and identification of Salmonella. A total of 140 stool samples from patients (83 males and 57 females) were collected at attended hospitals in Baghdad (Al-Yarmouk Teaching Hospital and Baghdad Teaching Hospital) and cultured on selenite broth for enrichment and selectivity, then cultured on Salmonella-Shigella agar (HiMedia Laboratories Private Limited), a selective and differential medium.

Antibiotic susceptibility testing for Salmonella and Enterobacter. The Kirby-Bauer disc diffusion method (16), was used to conduct the antibiotic sensitivity test, and according to the Clinical and Laboratory Standards Institute (CLSI) (17), results were obtained for six different antibiotics (gentamycin, ciprofloxacin, azithromycin, tetracycline, cefotaxime and chloramphenicol) as follows: In sterile plates, Mueller-Hinton agar (HiMedia Laboratories Private Limited) was prepared and added. To create a moderate turbidity of bacterial suspension compared to the typical turbidity solution (McFarland standard 0.5), 3-5 colonies of bacteria were moved into a tube containing 5 ml normal saline, which roughly equates to $1.5x10^8$ CFU/ml. The plates were labelled. A cotton swab (sterile) was dipped into the inoculums and used to apply the bacterial suspension to Mueller-Hinton agar medium. By gently pressing and rubbing the swab on the tube's side above the liquid level, the remaining material was eliminated. The surface of the medium was rubbed with the swab three times, with the plate turning at a 60˚ angle. The swab was then wound around the border of the agar surface. The cover was closed and the inoculums were allowed to dry for 5-10 min at room temperature. With sterile forceps, the antibiotic disc was picked out, placed on the inoculation plate (each plate contains four discs), and then gently pressed on the agar to ensure it came into contact with the agar. After 30 min, the plates were turned upside down and incubated for 18 to 24 h at 37˚C.

Detection of bacteriocin (klebicin) in K. pneumoniae isolates. The presence of the klebicin gene in 32 isolates of *K. pneumoniae* was detected. DNA extraction was carried out for 32 *K. pneumoniae* isolates using the OneTaq® 2X Master Mix kit (New England Biolabs, Inc.). From the isolated DNA, the klebicin gene cluster was amplified using the following primers: Forward, 5'-CATTAGCGTCCGCAGAACAAG-3' and reverse, 5'-GCCGACAGAGTAAAACCTCCA-3' (designed for the present study; the primers were designed using Geneious prime 2023.1.1 software depending on a reference sequence from GenBank with the accession no. CP026155). The 16SrRNA gene was also amplified using the following primers: Forward, 5'-GGACGGGTGAGTAATGTC-3' and reverse, 5'-TCTCAGACCAGCTAGGGATCG-3' (18). The reaction mixture of PCR contained 2 μl of each forward and reverse primer (10 mM), 3 μl template DNA, 12.5 μl Green master mix, and 25 μl free nuclease water. The conditions for PCR were as follows: 10 min at 94˚C (the initial denaturation temperature); 32 cycles with 1 min at 94˚C (the denaturation temperature), 40 sec at 54˚C (the annealing temperature), and 2 min at 72˚C (the extension temperature); and 5 min at 72˚C (the final extension temperature). Electrophoresis with 1% agarose gels stained with RedSafe™ Nucleic Acid (Promega Corporation) was used to resolve the amplified DNA products. The ability of 32 isolates to produce bacteriocin (klebicin) was examined to detect the presence of the klebicin gene.

Extraction of klebicin from producing isolates. Klebicin was extracted from the *Klebsiella* isolates and the crude extract of Klebicin was obtained as follows (19): The bacterial isolates of *Klebsiella* which were cultured overnight in 2.5 ml of LB (HiMedia Laboratories Private Limited) was used to inoculate 100 ml sterile Luria Bertani broth (HiMedia Laboratories Private Limited) accompanied by 5% glycerol in a shaker incubator. Using a cooling centrifuge set at 5,000 x g for 30 min (temperature, 4˚C), the supernatant was separated. For the klebicin antibacterial activity test and protein analysis, the supernatant was used. The Bradford technique was used to estimate the protein content in the crude extract of klebicin (20) and the calculation of the protein concentration

was by assessed using the bovine serum albumin (BSA) (CDH Fine Chemical) standard curve.

Determination of the klebicin minimal inhibitory (MIC) concentration. The resazurin-based turbidimetric assay was employed for estimating the MIC (21) by preparing (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.09%) from the stock 100% of klebicin extract. On a 96-well microtiter plate, an aliquot of 100 μl double-strength Muller-Hinton broth (HiMedia Laboratories Private Limited) was added from the first to the 12th well in each row. Each first well of the microtiter plate received 100 μl of the klebicin extract, which was then pipetted in and mixed with the broth. The mixture was then moved 100 μl from the first well to the second well and carefully stirred. Up until the eleventh well, dilution was ongoing. Subsequently, 100 μl were removed and discarded from the eleventh well. The 12th well of each row served as a positive control (a control well devoid of klebicin extract). To all but the 11th well, 20 μl of an overnight diluted bacterial suspension that had its turbidity corrected to the 0.5 MacFarland standard was added and thoroughly mixed. The 11th well hence acted as a negative control. A total of 5 μl resazurin (Abcam) (6.75 mg/ml) were added to each well followed by incubation at 37˚C for 18-24 h. Blue to pink color shifts were observed and noted. Prior to the color change, the lowest concentration was found to be the MIC.

Biofilm formation assay. A colorimetric microtiter plate technique was used to determine biofilm development quantitatively (22): The isolates were inoculated in brain heart infusion broth (HiMedia Laboratories Private Limited) and then incubated for 24 h at 37˚C. Subsequently, 100 μl bacterial growth and 2 ml ordinary saline were added to a tube, and the turbidity was adjusted to the McFarland standard of 0.5. A 180 μl of 1% glucose-containing brain heart infusion broth was added to sterile, 96-well polystyrene microtiter plates with flat bottoms. 20 μl of an adjusted turbidity bacterial suspension was placed in three wells of sterile flat-bottomed 96-well polystyrene microtiter plates. In total, six wells of bacterial-free brain heart infusion broth served as the negative control. The plates were not shaken during the 24 h that they were incubated at 37˚C under their covers. The plate was dried after three rounds of distilled water washing following incubation. Following incubation for 15 min at room temperature and the addition of 200 μl absolute methanol (Alpha Chemika) to each well, the biofilms were fixed by washing and air-drying the wells. The plates were stained for 15 min at room temperature using 200 μl of a 0.5% crystal violet solution (CDH Fine Chemical), washed three times in water, and dried for 30 min at 37˚C. A total of 200 μl glacial acetic acid (HiMedia Laboratories Private Limited) and 100% ethanol [Thomas Baker (Chemicals) Pvt. Ltd.] (1:1) were used to resolubilize the dye for 10 min. At 630 nm, the optical density (OD) of each well was determined using a microtiter plate reader (BioTek Instruments, Inc.; serial no. 130131A). It was found that the cut-off OD, or ODc, was three standard deviations higher than the mean OD of the negative control. All isolates were sorted into four categories based on the ODc value as follows: Non-producers, weak biofilm, moderate biofilm and strong biofilm, as shown in Table I.

Table I. Bacterial adhesion categorization on microtiter plates (23).

Mean OD630	Biofilm intensity
OD ≤ ODc	Non-adherent
2ODc > OD > ODc	Weak
4 ODc > OD > 2ODc	Moderate
OD > 4 ODc	Strong

ODc, three standard deviations greater than the mean OD of the negative control.

Table II. Susceptibility of *Salmonella* isolates to antibiotics.

Antibiotic	Sensitive isolates of *Salmonella* (%)	Resistant isolates of *Salmonella* (%)
Gentamycin	31.25	68.75
Tetracycline	46.87	53.12
Ciprofloxacin	68.75	31.25
Cefotaxime	25	75
Azithromycin	50	50
Chloramphenicol	65.6	34.3

Detection of the antibiofilm activity of the klebicin crude extract. Estimation of the antibiofilm activity of klebocin was achieved by testing it on 32 isolates of *Salmonella* and one *Enterobacter* (indicator isolates). The same procedure designated above in the biofilm formation assay section was followed for biofilm production, although 100 μl klebicin crude extract subMIC were added to each well. The plate was then incubated for 24 h at 37˚C. Following the incubation period, each well was rinsed with water and stained, and the absorbance at 490 nm was then assessed using an ELISA reader (BioTek Instruments, Inc.; serial no. 130131A).

Estimation of biofilm inhibition. The activity of klebicin crude extract as an antibiofilm was tested on 32 isolates of *Salmonella* and one *Enterobacter*, using a 96-well microtiter plate as previously described (24). The formation of biofilm was accomplished by the addition 100 μl of bacterial suspension (10^8 cells/ml), followed by a 24-h incubation period at 37˚C. The plate was incubated at 37˚C for 24 h with a growth-free medium as a control before the antibiofilm substance was added. The decrease in biofilm growth was calculated using the following formula (25):

$$\% \text{ Reduction} = \frac{Cotrol\ OD - Test\ sample\ OD}{Control\ OD} \text{x}100$$

Results

Isolation and identification of bacterial isolates. i) K. pneumonia: A total of 2 g negative isolates of bacteria were obtained from 120 specimens of wounds which were suspected to be *K. pneumoniae* after culturing on MacConkey's agar medium.

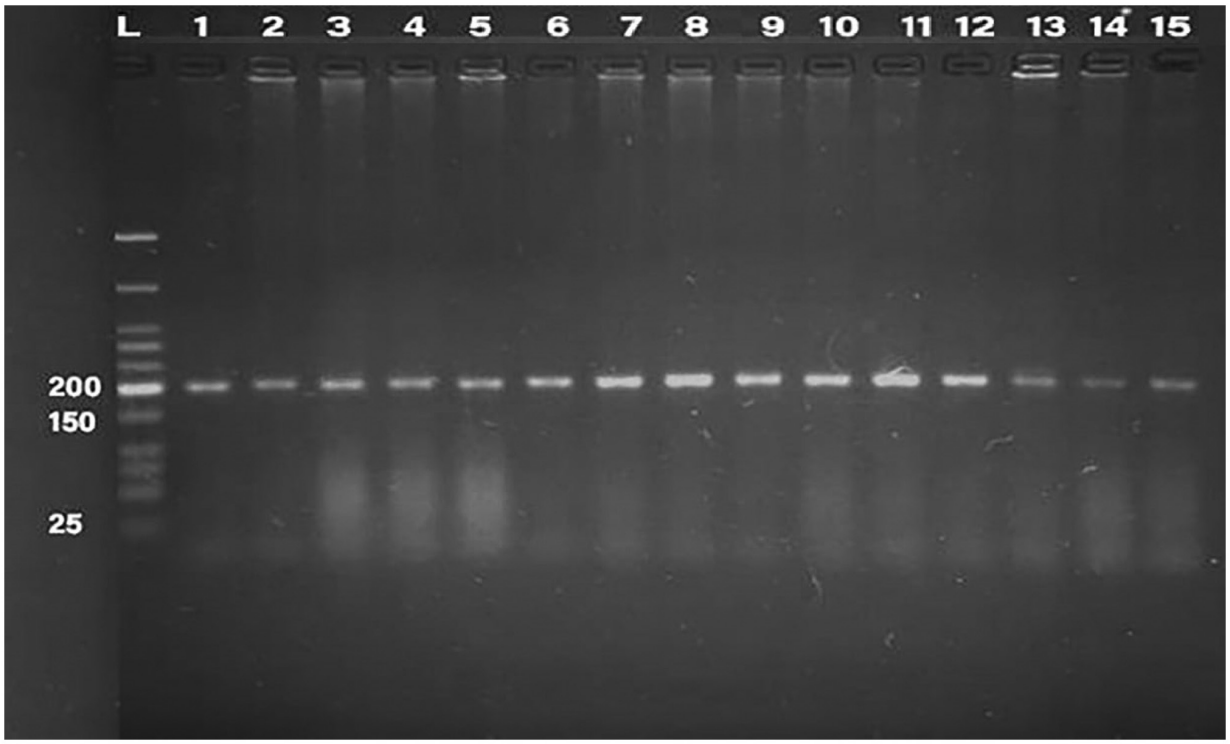

Figure 1. Agarose gel electrophoresis of the 16SrRNA gene of *Klebsiella pnuemoniae* (lanes 1-15) isolates. Lane L, 766 bp DNA ladder, 16SrRNA amplicon (198 bp). Electrophoresis was performed on a 1% agarose gel and OneTaq 2X, 100 volt for 90 min stained with red stain.

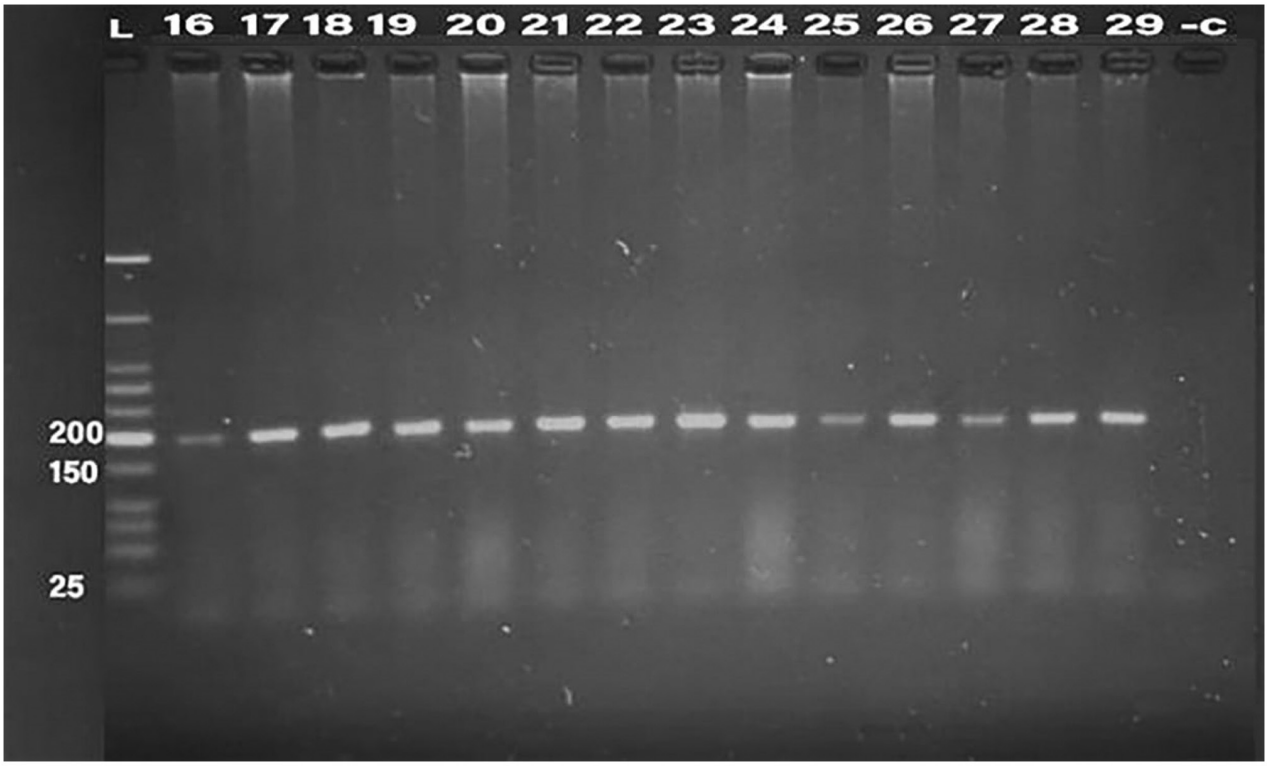

Figure 2. Agarose gel electrophoresis of the 16SrRNA gene of *Klebsiella pnuemoniae* (lanes 16-29) isolates. Lane L, 766 bp DNA ladder, 16SrRNA amplicon (198 bp). Electrophoresis was performed on a 1% agarose gel and OneTaq 2X, 100 volt for 90 min stained with red stain.

ii) *Enterobacter*: One bacterial isolate was obtained from wound specimens; iii) *Salmonella*: A total of 32 bacterial isolates were obtained from 140 stool samples.

Antibiotic susceptibility testing for Salmonella and Enterobacter. A total of six different antibiotics (gentamycin, ciprofloxacin, azithromycin, tetracycline, cefotaxime and

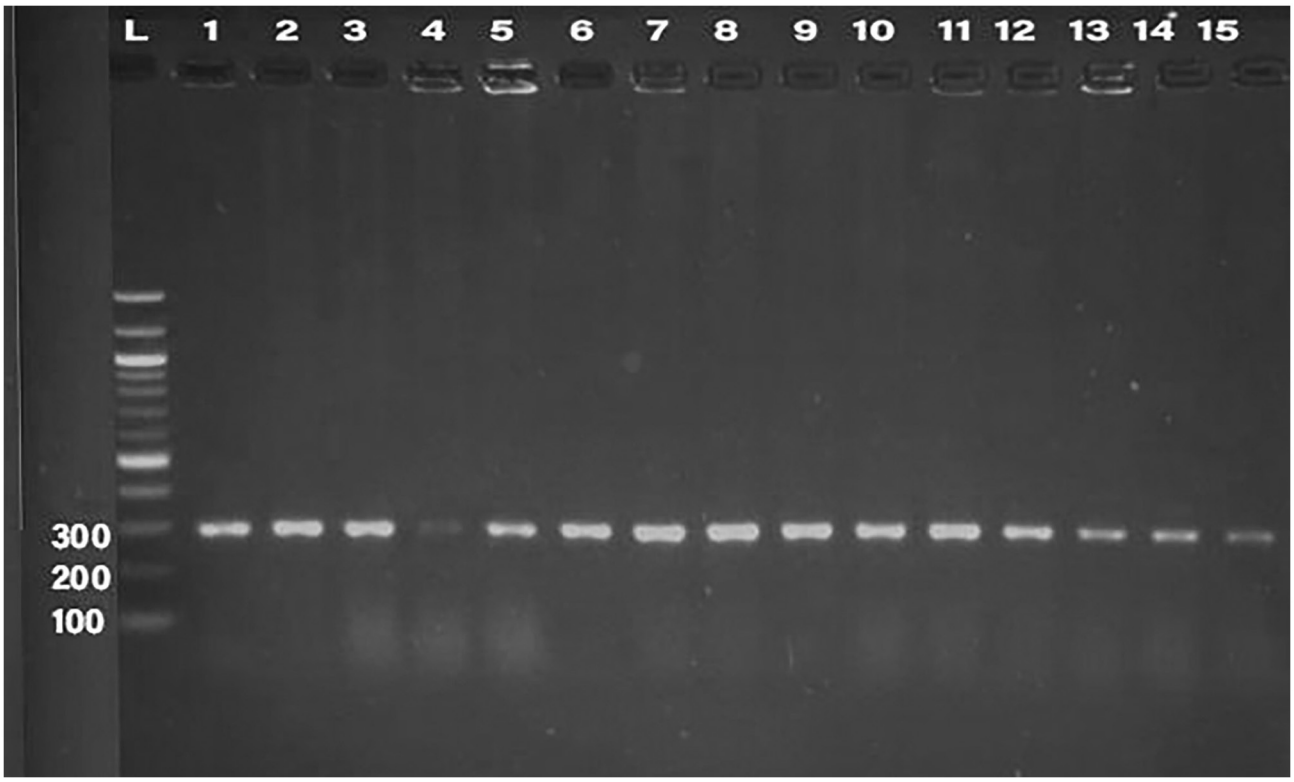

Figure 3. Agarose gel electrophoresis of the klebicin gene of *Klebsiella pnuemoniae* (lanes 1-15) isolates. Lane L, 1,517 bp DNA ladder, klebicin amplicon (294 bp). Electrophoresis was performed on a 1% agarose gel and OneTaq 2X, 100 volt for 90 min stained with red stain.

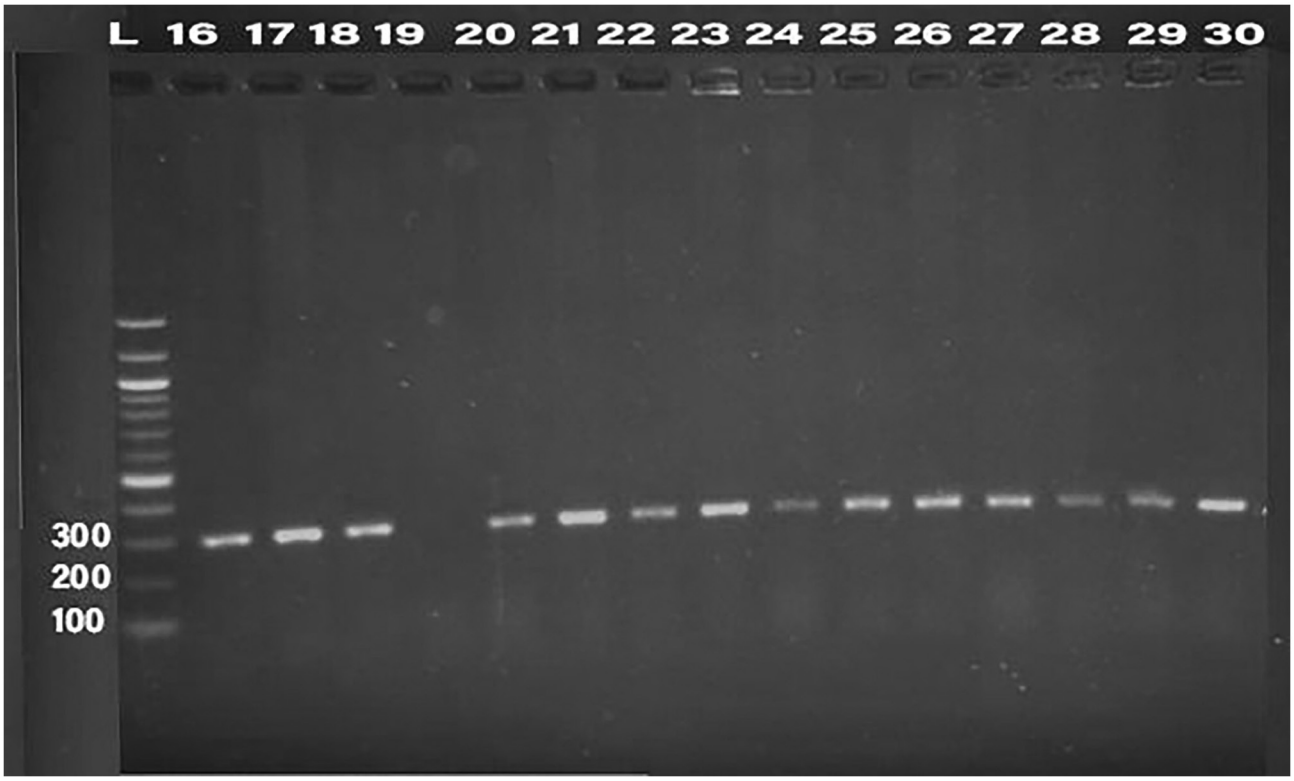

Figure 4. Agarose gel electrophoresis of the klebicin gene of *Klebsiella pnuemoniae* (lanes 16-30) isolates. Lane L, 1,517 bp DNA ladder, klebicin amplicon (294 bp). Electrophoresis was performed on a 1% agarose gel and OneTaq 2X, 100 volt for 90 min stained with red stain.

chloramphenicol) were used to assess the susceptibility of *Salmonella* and *Enterobacter* isolates towards them. The results revealed that the *Salmonella* isolates were resistant to gentamycin, tetracycline and cefotaxime with a resistance of

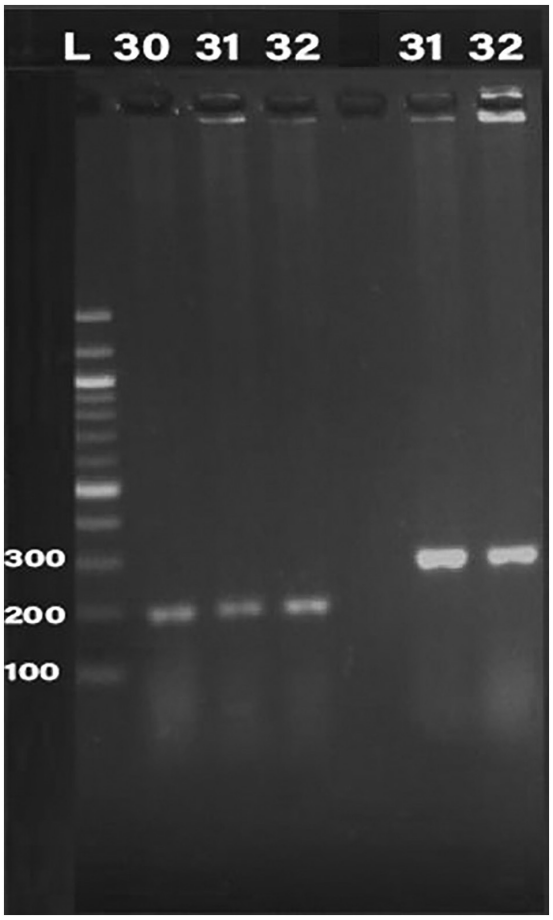

Figure 5. Agarose gel electrophoresis of the 16SrRNA gene of *K. pnuemoniae* (29,30,31) isolates and klebicin gene (31 and 32) isolates, L: 1517 bp DNA ladder, 16SrRNA amplicon (198 bp) and klebicin amplicon (294 bp). Electrophoresis was performed on a 1% agarose gel and OneTaq 2X, 100 volt for 90 min stained with red stain.

68.75, 53.12 and 75% respectively, while they were sensitive to ciprofloxacin and chloramphenicol with a sensitivity of 68.75 and 65.6%, respectively. Azithromycin affected 50% of the isolates, while 50% of them were resistant (Table II). *Enterobacter* exhibited resistance to all the antibiotics tested, while it was sensitive to ciprofloxacin only; 100% of the *Enterobacter* isolates were resistant to gentamycin, tetracycline, cefotaxime, azithromycin and chloramphenicol, and 100% of the isolates were sensitive to ciprofloxacin.

Detection of bacteriocin (klebicin) in K. pneumoniae isolates. PCR was conducted for 32 isolates, using primer of the 16SrRNA gene (198 bp) and klebicin gene (294 bp) for amplification; gel electrophoresis was used to confirm the bands, as presented in Figs. 1-5. The result revealed that the klebicin gene was detected in 31 (96.87%) of the *K. pneumoniae* isolates and it was not detected only in isolate 19.

Protein concentration in the klebicin crude extract. The protein concentration was calculated and the highest protein concentration was detected in the extract of K5, K10 and K17, as shown in Table III. The K17 isolate, which had the highest protein concentration in its extract, was used for further experiments in the present study.

Table III. Protein concentrations in the klebicin crude extract.

Klebsiella isolate	Protein concentration (μg/ml)	*Klebsiella* isolate	Protein concentration (μg/ml)
K1	140.455	K17	169.2953
K2	135.3679	K18	118.5249
K3	98.1706	K20	128.52927
K5	157.96899	K21	89.448
K6	102.5634	K22	131.6285
K7	168.745	K23	115.2993
K8	96.486	K24	99.318
K9	136.423	K25	124.4231
K10	165.4775	K26	135.6142
K11	115.5543	K27	130.6183
K12	138.467	K28	114.5521
K13	122.532	K29	122.7396
K14	117.341	K30	95.3185
K15	101.138	K31	140.4185
K16	132.3853	K32	138.817

Determination of the klebicin MIC against Salmonella isolates. The MIC of klebicin was assessed, and the results revealed that the *Enterobacter* isolate was inhibited by 50% of the klebicin extract, while the majority of the *Salmonella* isolates were inhibited by 50% of the klebicin extract, and only (S3, S10, S16, S20, S25, S28 and S32) were inhibited by 25% of the klebicin extract.

Antibiofilm activity of the klebicin extract against Salmonella and Enterobacter and their dual biofilm formation. Biofilm production was quantified using a colorimetric microtiter plate approach. The results revealed that prior to treatment with the klebicin extract, the *Enterobacter* isolate was a strong biofilm producer; however, following treatment, biofilm production became moderate. The majority of the *Salmonella* isolates were strong biofilm formers, while following treatment, most of them were moderate and weak biofilm producers, as shown in Table IV.

The results of dual biofilm formation by bacterial isolates of (*Salmonella* and *Enterobacter*) revealed that all of them were strong biofilm producers prior to treatment with the klebicin extract, while they became weak biofilm producers following treatment, as shown in Table V.

Estimation of biofilm inhibition. The use of the klebicin extract was obviously effective against biofilm reduction. In particular, it was more effective against dual biofilm formation by both bacteria, *Salmonella* and *Enterobacter*, compared to each bacteria alone Table VI.

Discussion

In order to preferentially separate Gram-negative and enteric (often found in the digestive system) bacilli and identify them based on fermenting lactose, MacConkey agar, a differential

Table IV. Biofilm forming capacity of *Salmonella isolates*.

Isolate no.	Mean value (before treatment)	Type of thickness	Mean value (after treatment)	Type of thickness
1	0.195	Very strong	0.1077	Weak
2	0.367	Strong	0.2369	Moderate
3	0.361	Strong	0.1534	Moderate
4	0.293	Strong	0.2194	Moderate
5	0.297	Strong	0.1898	Weak
6	0.302	Strong	0.2114	Moderate
7	0.377	Very strong	0.2763	Strong
8	0.378	Very strong	0.2456	Strong
9	0.243	Strong	0.2175	Moderate
10	0.534	Strong	0.1903	Moderate
11	0.537	Very strong	0.2923	Strong
12	0.394	Strong	0.2014	Moderate
13	0.361	Strong	0.1977	Moderate
14	0.590	Strong	0.2006	Moderate
15	0.284	Strong	0.2367	Moderate
16	0.401	Very strong	0.2449	Strong
17	0.185	Moderate	0.1742	Moderate
18	0.495	Strong	0.1155	Weak
19	0.372	Very strong	0.2771	Strong
20	0.502	Very strong	0.2425	Strong
21	0.225	Moderate	0.1742	Moderate
22	0.406	Strong	0.1162	Weak
23	0.403	Strong	0.2104	Moderate
24	0.503	Strong	0.1807	Moderate
25	0.453	Strong	0.2131	Moderate
26	0.527	Very strong	0.2776	Strong
27	0.283	Strong	0.2377	Moderate
28	0.351	Very strong	0.2755	Strong
29	0.286	Strong	0.2028	Moderate
30	0.496	Strong	0.184	Moderate
31	0.277	Very strong	0.2831	Strong
32	0.294	Strong	0.174	Moderate

and selective growth medium is used, and Gram-positive organisms are prevented from growing by crystal violet and bile salts (26). In the present study, this medium was used to isolate *K. pneumoniae* and *Enterobacter*. Isolates which were suspected to be *K. pneumoniae* were confirmed using VITEK-2 compact system. Following culture on MacConkey's agar medium, *Enterobacter* colonies were lactose fermented and motile compared with *K. pneumoniae* confirmed by VITEK- 2 compact system. *Salmonella* isolates were obtained following culture on selenite broth and transferred to Salmonella-Shigella agar that appears as pale colonies with a black center. Selenite broth, as a selective and enrichment medium, is used for the cultivation of *Salmonella* spp., that may be present in small numbers in the intestine and competing with its flora (27). The identification of isolates was confirmed using the VITEK-2 compact system.

Antibiotic susceptibility was performed to exhibit the high resistance rate in bacterial isolates, and resistance to more than one antibiotic was observed; thus, this leads to the necessity for alternative mechanisms to combat antibiotic resistance. A previous study reported that *Salmonella* isolates in high proportions were resistant to tetracycline (n=53.9%) and ciprofloxacin (n=47.2%) (28). Another study demonstrated that 98% of *Salmonella* isolates were non-susceptible to ciprofloxacin (29). Patil and Mule (30) reported that all isolates (100%) of *Salmonella* were sensitive to cefixime, ceftriaxone and azithromycin, and 94.4% (237/251) of the bacterial isolates were significantly sensitive to chloramphenicol.

In the present study, PCR was conducted for 32 isolates of *K. pneumoniae*, and the klebicin gene was found in the majority of these isolates, apart from one. In their study, Kareem *et al* (31) reported PCR amplification results. PCR products corresponding to the klebicin gene appeared in 15 isolates (48.39%) (31).

Herein, the protein concentration was determined in the klebicin crude extract of all *K. pneumoniae* isolates and the

Table V. Biofilm forming capacity of dual bacterial isolates (*Salmonella* and *Enterobacter*).

Isolate no.	Mean value (before treatment)	Type of thickness	Mean value (after treatment)	Type of thickness
1	1.054	Strong	0.073	Weak
2	1.065	Strong	0.079	Weak
3	1.057	Strong	0.075	Weak
4	1.054	Strong	0.331	Weak
5	1.068	Strong	0.080	Weak
6	1.079	Strong	0.076	Weak
7	1.060	Strong	0.076	Weak
8	1.068	Strong	0.074	Weak
9	1.057	Strong	0.081	Weak
10	1.071	Strong	0.077	Weak
11	1.067	Strong	0.077	Weak
12	1.072	Strong	0.084	Weak
13	1.065	Strong	0.072	Weak
14	1.068	Strong	0.089	Weak
15	1.061	Strong	0.078	Weak
16	1.086	Strong	0.088	Weak
17	1.062	Strong	0.071	Weak
18	1.080	Strong	0.088	Weak
19	1.063	Strong	0.070	Weak
20	1.070	Strong	0.075	Weak
21	1.035	Strong	0.072	Weak
22	1.074	Strong	0.082	Weak
23	1.078	Strong	0.080	Weak
24	2.970	Strong	1.973	Weak
25	1.042	Strong	0.068	Weak
26	1.088	Strong	0.077	Weak
27	1.075	Strong	0.084	Weak
28	1.066	Strong	0.077	Weak
29	1.085	Strong	0.089	Weak
30	1.079	Strong	0.087	Weak
31	1.075	Strong	0.084	Weak
32	2.405	Very strong	1.514	Strong

isolate K17 with the highest protein concentration extract was used to perform the ensuing analyses. Bacteriocins are ribosomal proteins or peptides. When bacteriocin-producing bacteria release bacteriocin, this can interact with the appropriate receptor on the surface of the vulnerable bacteria to kill it (32).

Klebicin crude extract of the K17 isolate had an inhibitory effect on *Salmonella* isolates with two MICs. The sensitive bacteria are killed by klebicin via a number of mechanisms. Klebicin binds to certain receptors, which are outer membrane proteins that are used for the entrance of various nutrients. Subsequently, either the Tol or TonB systems transport klebicin over the periplasm and through the outer membrane (33). The action of klebicins would include either creating a channel (voltage-dependent) into the inner membrane or by using their activity of endonuclease on DNA, rRNA, or tRNA to reach their target (34,35). Klebicins are proteins that are encoded by both chromosomes and plasmids (14).

Klebicin crude extract markedly affected the biofilm formation of *Salmonella* and *Enterobacter* isolates and reduced it. High percentages of inhibition were observed in the dual biofilm formation with two types of bacteria. Khalaf and Hussein (36) reported that klebicin crude extract affected the formation of biofilm in some bacterial isolates, such as *Klebsiella*, *Proteus* and *E. coli*.

To date, only a limited number of studies have examined the effects of klebicin on bacteria and their biofilm development compared to other types of bacteriocins produced by other bacteria, such as colicin from *E. coli* and pyocin from *Pseudomonas*. Colicins, which are derived from *E. coli,* and other bacteriocins that are similar to colicins, which are derived from a variety of Gram-negative bacteria, are poisonous to bacterium closely similar to the strain that produces it. Of note, >90% of *Pseudomonas aeruginosa* strains generate pyocins, and each strain is capable of producing several pyocins. The pyocin genes are found on the chromosome of

Table VI. Biofilm reduction of the *Salmonella* and the combination of the *Salmonella* and *Enterobacter* isolates following treatment with klebicin extract.

Salmonella isolates	Biofilm reduction following treatment with klebicin extract (%)	*Salmonella* and *Enterobacter* isolates	Biofilm reduction following treatment with klebicin extract extract (%)
S1	44.7	S1 + E	93
S2	35.4	S2 + E	92.5
S3	57.5	S3 + E	92.9
S4	25.1	S4 + E	68.5
S5	36	S5 + E	92.5
S6	30	S6 + E	92.9
S7	26.7	S7 + E	92.8
S8	35	S8 + E	93
S9	10.4	S + 9E	92.3
S10	64.3	S10 + E	92.8
S11	45.5	S11 + E	92.7
S12	48.8	S12 + E	92.1
S13	45.2	S13 + E	93.2
S14	66	S14 + E	91.6
S15	16.6	S15 + E	92.6
S16	38.9	S16 + E	91.8
S17	5.8	S17 + E	93.3
S18	76.6	S18 + E	91.8
S19	25.5	S19 + E	93.4
S20	51.6	S20 + E	92.9
S21	22.5	S21 + E	93
S22	71.3	S22 + E	92.3
S23	47.7	S23 + E	92.5
S24	64	S24 + E	33.5
S25	52.9	S25 + E	93.4
S26	47.3	S26 + E	92.9
S27	16	S27 + E	92.1
S28	21.5	S28 + E	92.7
S29	29	S29 + E	91.7
S30	62.9	S30 + E	91.9
S31	5.3	S31 + E	92.1
S32	40.8	S32 + E	37.1

Pseudomonas aeruginosa (37). S2-pyocin and antibiotics were tested against *P. aeruginosa* biofilms *in vitro* in the study by Smith *et al* (38). S2-pyocin was shown to be the most effective against *Pseudomonas aeruginosa* biofilms, resulting in a 4 log decrease in *Pseudomonas aeruginosa* survival (38). Another study revealed that *K. pneumoniae* clinical isolates were capable of producing bacteriocin (klebocin) which affected pathogenic isolates, such as other *Klebsiella*, *E. coli* and *Proteus* (14). Klebocin had a strong antibacterial and antibiofilm action. Therefore, it was concluded that these outcomes may be a potential source of antimicrobial agents (14). The heterologous action of klebocins on numerous pathogenic species of Gram-negative and some Gram-positive bacteria, those isolated from individuals with persistent otitis media and pyelonephritis, in particular, may suggest that these klebocins can be used as an alternative to broad-spectrum antibiotics (39).

In the present study, the effects of klebicin crude extract on *Salmonella* and *Enterobacter* isolates was notable according to the data obtained, particularly on biofilm formation reduction, since the current low efficacy of antibiotics towards bacterial infections caused by biofilms can be largely attributed to the fact that the biofilm mode of development is characterized by prolonged infection by resistant pathogens; therefore, antibiotic alternatives should be researched. The data obtained herein suggest the possibility of the use of bacteriocin (klebicin) as an antibacterial and antibiofilm agent. Further studies need to be conducted to purify and characterize klebicin, examine its effects on biofilm formation and examine it under an electron microscope.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NSA and HKT contributed to the conception and design of the study. All authors (NSA, HKT and AHO) were involved in the study methodology. NSA and HKT contributed to the data collection and analysis. NSA was involved in the writing of the manuscript. NSH and HKT confirmed the authenticity of all the raw data. All authors have read and agreed to the published version of the final manuscript.

Ethics approval and consent to participate

The protocol for the present study was approved by the Ethics Committee at the Department of Biology (University of Baghdad) and the Iraqi Ministry of Health (Reference: CSEC/0323/0056). Written informed consent was obtained from all the patients. The study was carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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