Phenotypic and genotypic detection of microcin produced by some *Enterobacteraceae*

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Abstract

Back ground: Microcins are ribosomal synthesized antimicrobial peptides produced principally by bacteria of the *Enterobacteriaceae* family that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) that share the same ecological niche.

Objective: The ability of the *Enterobacteriaceae* to produce microsines.

Materials and methods: One hundred urine samples have been collected from patients suffering from UTI whom admitted to Al Hakim Hospital in AL-Najaf City during the period from 17October 2017 to 13 February 2018.

Results: The results of PCR technique for amplification show that all isolates were possess *mic N* by appearance of amplicon with molecular weight 938 bp while 46 (76%) isolates were possess *mic N1*and 24 (40%) isolates were possess *mic N2* gene by appearance of amplicon with molecular weight 368 bp and 167 bp respectively.Cross-streak activity assay was used to detect the selected strains with antibacterial activities against pathogenic bacteria.

Conclusion: some isolates have one or more gene microcin encoded of , however, microcin N is present in all isolates.

Keywords: *Enterobacteriaceae*, Microcin, PCR.

Introduction

The family *Enterobacteriaceae* comprises a large heterogeneous group of Gram-negative, although they are uncommonly found outside the gastrointestinal tract, they are a leading cause of nosocomial disease (1,2). *Enterobacteriaceae* are wide world distributed, it might be found in, plants, soil ,water, humans and animals, because it is responsible for a broad range of infections in humans and animals. the main genera are *Escherichia*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Shigella* and *Yersinia*. Certain species are part of the normal flora of animals including humans, although many are frequently associated with diarrheal disease and extra intestinal infections.(3). Bacteria engender antimicrobial compounds to inhibit or kill other competing strains because they survive in the environment and their competition with other microorganisms for purse.(4) .This antimicrobial peptides are just one of the many classes of antimicrobial component, One of these peptides, microcin N are low-molecular-weight (10 000 Da) antimicrobial peptides secreted by members of the *Enterobacteriaceae* family and are associated in microbial competition inside the intestinal tract. Microcin, which is widely distributed in enterobacteria, is a small ribosomally-synthesized, powerful antimicrobial peptide against a wide range of Gram-negative and some Gram-positive bacteria. (5) As a hopeful antibiotic against antibiotic resistance, this study is aimed to determine the presence of gene encoding microcin N in *Enterobacteriaceae* isolated from patients suffering from UTI as well as phenotypic detection of microcin by Cross-streak activity assay.

Methods

Sampling and Bacteria Isolation

A total of 100 specimens were collected from patients with urinary tract infection of both sex with age group range from 20-55 years old whom admitted to Al Hakim Hospital during the period from 17 October 2017 to 13 February 2018. To identified bacterial isolates they were re-cultured on nutrient agar media for identification then each purified single colony was cultured on Chrom agar to certain identification of bacterial isolate. Urine samples

were diluted with normal saline (5ml), then transferred by a sterile loop to Chrom agar media where they were streaked and incubated at 37˚C for 24-48 hr for isolation of *Enterobacteriaceae* .

Table (1): Identification of Bacterial Isolates on Chrom agar media

Name of bacteria	Result	
	+Ve	-Ve
E.coli	**Dark pink**	
Enterobacter	**Metallic bule**	
Citrobacter	**Metallic blue with red halo**	
Enterococcus	**Turquoise bule**	
Staphylococcus aureus	**Golden, opaque, small**	**no color**
Staphylococcus saprophyticus	**Pink, opaque, small**	

DNA Extraction

Boiling methods (TE buffer method) that described by (6) was carried out was carried out using the Template DNA that was prepared by dissolving 1µl of bacteria in 1ml of TE buffer, centrifuged at 5000 rpm for 5 min, and the pellet resuspended in 100µl of TE buffer. The suspension was boiled at 100c◦ for 10 min before centrifugation at 5000 rpm for 5 min. The supernatant accommodated as PCR template. Extracted DNA was qualified by using 1.5 % agarose gel electrophoresis.

Preparation of Primer

The sequences of oligonucleotide were mentioned in (Table 2). All primers that used in this study were prepared according to the recommendation of manufacture by dissolving lyophilized primer with a propriate TE buffer to yield 100 µM of stock solution. Working solution was prepared by diluent with TE buffer to yield 1µM of each primer.

Table (2): The sequences of oligonucleotide, primer that used

Genes	Primer sequence	Size of amplicon	
micN	**F- TCGGAGCGATTATGGACAGC**	**983**	**Designed in this study**
	R- TCTGGCACATTCCACCATCC		
***micN*-1**	**F- TACGGCATTATCAGCCGGAC**	**386**	
	R- TCAAGAGAACGACCACCTGC		
***micN*-2**	**F- GAAAGCCAGATGCAGACCCT**	**167**	
	R- CCCGGTGTAACACTCAACGA		

Amplification Process

Amplification reaction mixture was prepared with final volume 20 µl by mixing all contents in Table (3) and Table (4), then centrifuged for short spin and transferred to thermo cycler, with the following thermal cycling conditions: 2 minutes at 94°C and 30 cycles of amplification consisting of 30 seconds at 94°C, 30 seconds at 59°C, and 60 seconds at 72°C, with 5 minutes at 72°C for the final extension. PCR products were analyzed by electrophoresis on a 1% agarose gel at 70V for 60 minutes. Gel documentation system was used for ritualization of amplicon bands.

Table (3): The Mixture of Amplification Reaction

Item	Volume µl
PCR Master mix	5
Forword	1
Reverse	1
DNA template	2
Nuclease free water	11
Total volume	20

Table (4): The Amplification Condition

Gene	Initial Denaturation (ºC/min)	No. of Cycle	Denaturation (ºC/sec)	Annealing (ºC/ sec)	Extension (ºC/ sec)	Final extension (ºC/min)
micN	95/2	30	95/30	59.3/30	72 /40	72/5
micN1	95/2	30	95/30	59.1/30	72/100	72/5
micN2	95/2	30	95/30	59.3/30	72 /20	72/5

Phenotypic detection of microcin

To evaluate the ability of all bacterial isolates to produce microcin, a method described by (7) was carried out with some modifications. M9 and M63 media were used to evaluated the efficiency of bacterial isolates to produce microcin. All bacterial isolates were streaked on M9 and M63 media as a width line in the middle of media and incubated for 24 hr. at 37˚C. At the terminus of incubation period, the densely overgrown streak was chloroform-inactivated utilizing pipette covering the whole streak of bacteria by thin film of chlorophorm. The inhibitory activity was tenacious qualitatively by applying 10 µl of a standardized 0.9% NaCl suspension of tested microcin that engendered from isolate. A result was defined to be positive if an inhibition zone was present on both side of the engendering strain and inhibitory activities were visually relegated as vigorous, clear and visible.

Detection the activity of purified microcin

The activity of purified microcin was detected with one exception that the growth of bacterial isolate were replaced by microcin (0.5 gm/ml) which streak on the surface of M63 medium and pathogenic isolate were streak vertically on microcin, then the plate were cultured at 37°C for 24 hr. The appearance of inhibition zone referred to activity of microcin.(8).

Purification of microcin

Microcin purification method was carried out as described by (9).

Results and Discussion

The result of isolation and identification of *Enterobacteriaceae* members on CHROM Agar showed a wide distribution of *Enterobacteriaceae* members among all collected sample in which all 100 (100%) sample gave +ve culture .

Enterobacteriaceae, reported by (10) were recovered from pus samples 44.19 %, followed by urine 36.05% and blood 8.14% (6) showed that46.1% *Enterobacteriaceae* from patients with diarrhea , 42.3% animals and insects. (11) collected from faecal samples of inpatients. A number of studies have been confirmed *Enterobacteriaceae* bacteremia most commonly occurred in patients with malignancies 48.9% or hepatobiliary stones 22.2%.

The percentage of isolation of *Enterobacteriaceae* species was as followed: 48% (48 isolate) of *E. coli* ; 32% (32 isolate) of *Klebsiella* ; *Citrobacter* isolates 9 (9%) ; 11% (11 isolate) of *Enterobacer*.

Microcin antibiotic like structure which produced by a member of *Enterobacteriaceae* and promising antibacterial activity against antibiotic resistance bacteria and other closely related microorganism (12).The result showed that the minimal M9 and M63 were more efficient in enhancement of bacterial isolate to produce microcin in which a large inhibition zone (Figure 1).

Other studies showed that microcin may be produced during stationary phase except microcin E492 which produced during exponential phase (13). In the present study we found that microcin was produce during stationary phase (after 24 hr of incubation). This variation in the time of production may correlated with the structure of microcin which is a thermo stable peptides protease resistance and resistance to extreme PH (14). Many studies showed that the activity of microcin correlated with the structure of outer membrane of bacterial cells (a target for microcin activity).(15, 16).

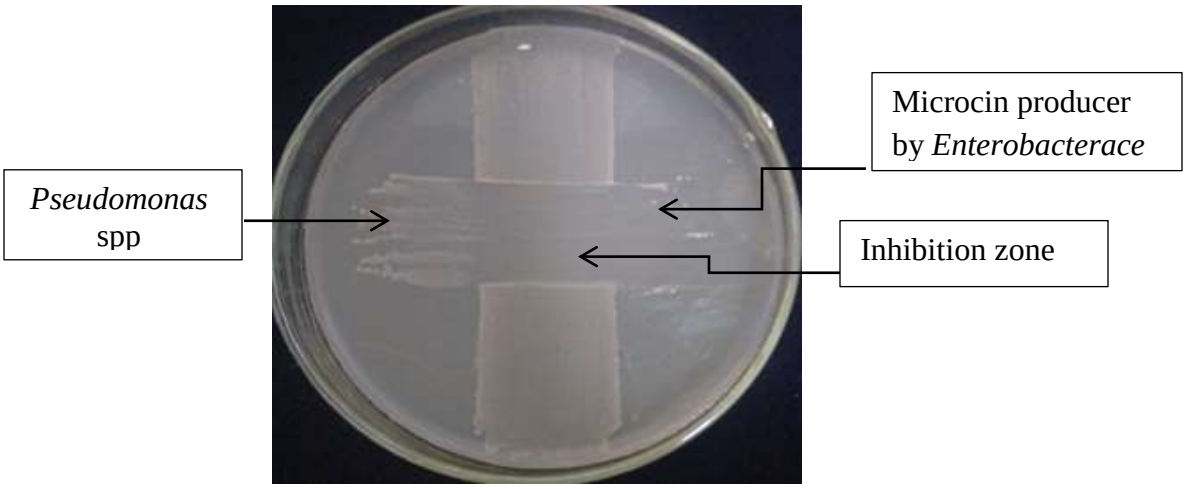

Figure (1): inhibitory activity of pathogenic bacteria (*Pseudomonas*) microcin against *Enterobacterace*, measured with the cross-streak activity assay

In this study, microcin N gene from *Enterobacteriaceae* was screened by using PCR in 100 clinical specimens of patients. Sequencing confirmed the fidelity of amplicon. Results showed a high prevalence of *Enterobacteriaceae* strains harboring this gene, which can be an explanation of these bacteria being a common microbial flora in some of AL-najaf hospital. More experiments and research in the For example, the study was done by (4) the effect of microcin produced by *Enterobacteriaceae* on *E. coli* in a co-culture condition confirmed the sensitivity of *Escherichia coli* to microcin (17). Hindered isolate clinical specimens were collected from urinary tract infection and were selected for genotypic detection of gene coding for microcin. These isolate were selected on its microcin activity and site of isolation. The results of agarose gel electrophoresis rises of amplicon showed that all isolate

were possess mic N by appearance of amplicon with molecular weight 938 bp while 33 (33%) isolates were possess *mic N1*and 21 (21%) isolates were possess *mic N2* gene by appearance of amplicon with molecular weight 368 bp and 167 bp respectively (Figure 2,3,4). because of the high importance of microcin N in medicine, we focused on its prevalence in bacteria isolated from hospitals. Past and ongoing researches on antimicrobial peptides have shown that these compounds have a great potential to be used in food and medical industries. Discoveries of new antimicrobial peptides and the understanding of the biological process involved in the synthesis, immunity, and regulation of antimicrobial peptides, should play a role in this field, with emphasis on practical applications in the industry (3).

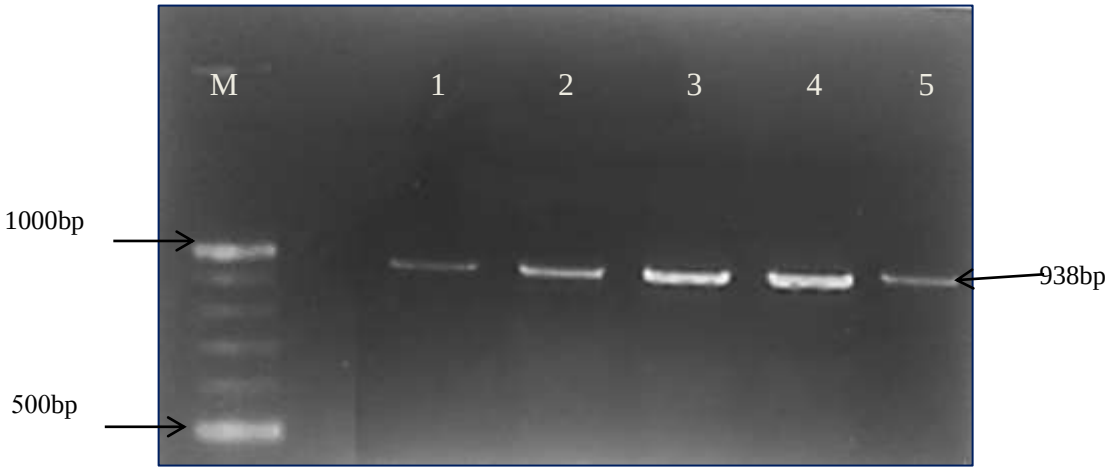

Figure (2): Agarose gel electrophoresis (1%at 70 volt 60 min) of amplicon resulted from amplification of mic N (938 bp). Lane L:DNA, Marker (1000 bp) lane (1, 2, 3,4, 5) positive to for amplification

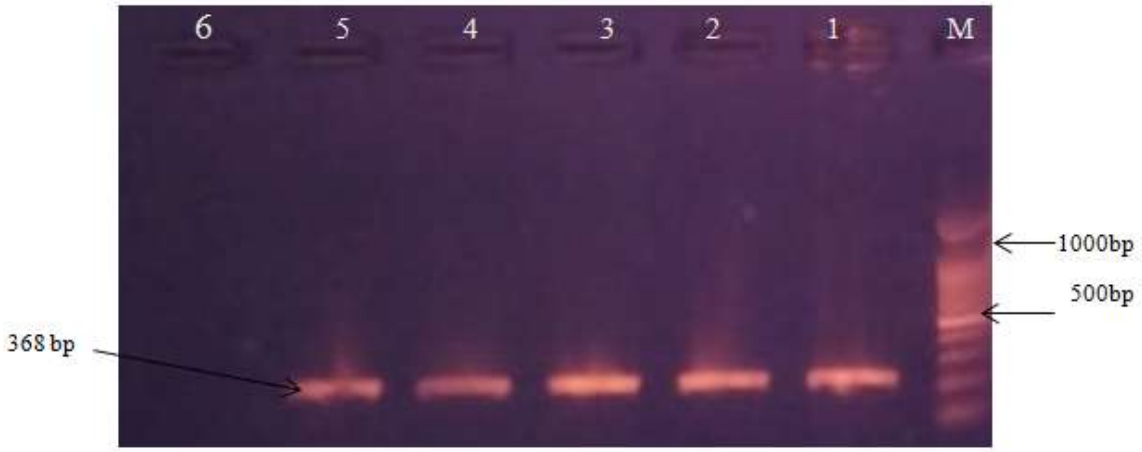

Figure (3): Agarose gel electrophoresis (1%at 70 volt 60 min) of amplicon resulted from amplification of mic N1 (368 bp). Lane L:DNA, Marker (1000 bp) lane (1, 2, 3,4, 5) positive to for amplification, Lane (6) negative for amplification

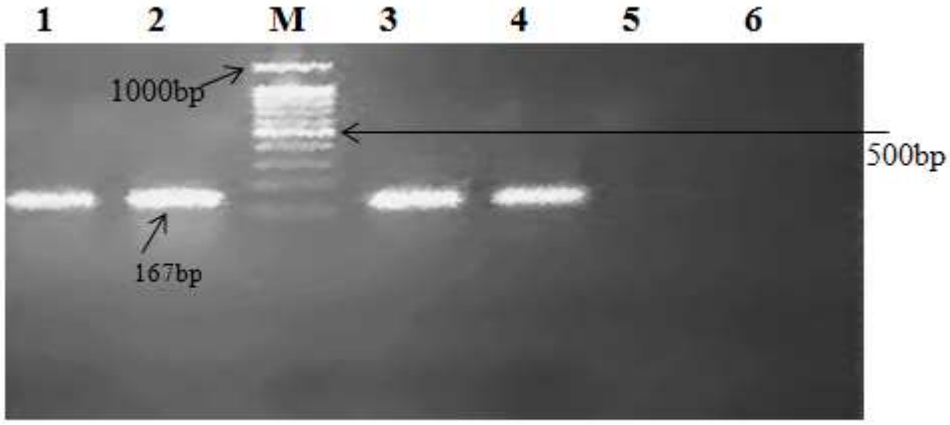

Figure (4): Agarose gel electrophoresis (1%at 70 volt 60 min) of amplicon resulted from amplification of *mic N2* (167 bp). Lane L:DNA, Marker (1000 bp) lane (1,2,3 ,4) positive to for amplification, Lane (5,6), negative for amplification

Conclusions

1- Microcin was detected by molecular technique, it is showed that some isolates have one or more gene microcin encoded of, however, microcin N is present in all isolates.

2- Microcin production is seen when minimal media M63 is used**.**

3- All isolates showed high ability to activity against pathogenic isolate.

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