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Molecular Detection of locally *Bacillus cereus* isolated from Bovine milk

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ABSTRACT

One hundred sample of bovine milk were collected from different area in Baghdad city/Iraq, and cultured on the selective medium, Mannitol Egg Yolk Polymyxin Agar (MYP), for isolation of *Bacillus cereus*, biochemical tests were carried out for identification of *B.cereus* colonies then confirmed by API 20E and Vitek 2 systems, 15 (15%) isolates has been diagnosed as *B.cereus* and identified with PCR technique used specific primer of 16SrRNA with 676 bp. Three different bacterial strains *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were isolated from bovine milk with percentage of (60%), (80%) and (40%) respectively used as target bacteria. The 15 *B.cereus* isolates designed as (BC1- BC15) tested for their ability to produce bacteriocin as antimicrobial activity against target bacteria the results showed that all the (15) isolates were able to produce bacteriocin with percentage 100%, one isolates (BC4) was selected which showed the largest inhibition zone diameter against target bacteria (14mm) against *S.aureus* (8mm) against *E.coli* and (6mm) against *P.aeruginosa*, Sequence analyses have been determined for (BC4) isolates. The protein concentration of bacteriocin was (0.27 mg/ml).

Keywords: *Bacillus cereus*, 16SrRNA, Bacteriocin, Bovine milk.

1. INTRODUCTION

The genus *Bacillus* is a large and diverse group of bacteria belonging to the family *Bacillaceae*, Phylum Firmicutes which is encompassing more than 60 species, from these species *Bacillus cereus* which is gram-positive rods often arranged in pairs or chains with rounded or square ends and usually have a single endospore [1].

B. cereus has a circular chromosome measuring (5,411,809 nt) in length. The genome structure of *B. cereus* consists of 5481 genes, 5234 protein coding, 147 structural RNAs, and 5, 366 RNA operons [2] and has a diverse range of plasmids that vary in size from 5 to 500 kb. In the present study A polymerase chain reaction assay for the detection of all members of *B. cereus* group, which targets 16S rRNA, has been developed by [3] which has been proven to be used as a powerful tool for phylogenetic identification of *Bacillus cereus* from closely related species [4]. The spectrum of potential *B. cereus* toxicity ranges from strains used as probiotics in animal feed to highly toxic strains already reported to be responsible for fatalities. *B. cereus* is capable of producing different nonspecific virulence factors including cell surface proteins, cytotoxic components and degradative enzymes of these proteases, phospholipases, haemolysins and enterotoxins [5] and also used in many medical, pharmaceutical, agricultural, and industrial processes that take advantage of their wide range of physiologic characteristics and their ability to produce antibiotics, and other metabolites such as bacteriocin which is called (cerein) which are ribosomally-synthesized proteins, such as cerein 8A produced by *B. cereus* 8A [6], which are antagonistic to other bacterial strains, interferes with cell membrane integrity and causes multiple pores in the cell membranes resulting cell death [7].

This study was conveyed to help screen for Iraqi bacteriocin-genic *B. cereus* strain which identified by molecular technique from bovine milk with their activity against undesirable bacteria, such as pathogens and spoilage organisms.

2. MATERIALS AND METHODS

2.1 Samples collection and their sources

One hundred samples of bovine milk were collected randomly with sterile container from different area in Baghdad city/ Iraq, from October 2016 to January 2017, and kept in ice box and transferred immediately to the laboratory to avoid any external contamination and multiplication of microorganisms [8].

2.2 Isolation and identification of *B.cereus*

A volume of 10 ml of milk sample placed in the water bath at 80 °C for 12 min, to destroy vegetative bacteria and fungi and to make easier for isolation, then cultured on MYP agar and blood agar**,** the suspected colonies were identified with microscopic examination stained with Gram and Spore stain and performed biochemical test and confirmed with API20 E and Vitek2 system [8].

2.3 Isolation of target bacteria

For the isolation of target bacteria (***Escherichia coli***, ***Staphylococcus aureus***, ***Pseudomonas aeruginosa***) from bovine milk sample and streaked on the surface of selective media for each microorganism, the suspected isolates were identified with microscopic examination and biochemical test finally confirmed with API Staph and API20E system. The strains were maintained on Brain heart infusion agar slants [8].

2.4 Molecular detection of isolated *Bacillus cereus* by PCR

2.4.1 DNA extraction

DNA for PCR amplification was extracted from isolated *B.cereus* by used G-spin ™ Total DNA Extraction Kit (INtRON, korea). One or two ml of bacterial culture was transferred to a 2 ml micro-centrifuge tube then Centrifugation for 1 min. at 13.000 rpm**,** Lysozyme buffer was added to the centrifuge tube then vortex to completely dissolve the lysozyme, after lytic was completed, centrifuge for the second time and washed with buffer, cell transferred to spin column, then eluted the extracted DNA with buffer and stored at 4°C. The purified DNA was detected by electrophoresis in 1 % agarose gel, 5 µl of DNA was mixed with 3 µl of loading dye (bromophenol blue) and visualized by U. V. light [9].

2.4.2 Amplification genes encoded for 16S rRNA of *B. cereus*

A specific primer was used for PCR amplification of 16S rRNA gene: F1: 5'– GCGGCGTGCCTAATACATGC-3' and R1: 5'–CTCTACGCATTTCACCGCTAC-3'. Used master mix which contains (Taq polymerase, PCR buffer, MgCl2 and dNTPs) and the final concentration of primers was 10 pmol/µl with TBE buffer, PCR was performed under following conditions: the Initial Denaturation was 94°C for 3min followed by denaturation at94°C for 30 sec, annealing at 56°C for 30 sec for 30cycle, the amplified sample was directly loaded in a 2 % agarose gel and for 1:30 h, then visualized by U.V Light [10].

2.4.3 Nucleotide Sequences of 16SrRNA Gene

Forward primer of 16SrRNA was sent to Korea for identifying sequences product PCR, the data of the nucleotide sequence of the 16S rRNA gene of *B.cereus* isolates were aligned and compared with similar sequences of the 16S rRNA gene of the reference strains of *B.cereus* in GenBank searched in the BLAST program of the NCBI website [10].

2.5 Antimicrobial Activity of *B.cereus* bacteriocin against target Bacteria

Extraction of Bacteriocin

Bacillus cereus was grown in 25 ml BHI broth and incubated aerobically at 30 °C for 18hrs with shaking, the growing cell were separated by centrifugation at (8,000 rpm for 15 min, 4ºC) and the culture supernatant was filtered through a 0.22-µm (Millipore), The cell free supernatant was used as crude bacteriocin, inhibitory activity of crude bacteriocin against sensitive strain was assayed according to [11].

2.6 Screening of *Bacillus cereus* bacteriocin as antimicrobial activity

Antimicrobial activity of bacteriocin against each indicator microorganisms (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) was determined by well diffusion method on Mueller-Hinton agar under aerobic condition incubated at 37 °C for 24 h-48h, the antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells, the strain which showed the maximum zone of clearance was chosen for further study [12].

2.7 Determination of Protein concentration

Protein concentration was determined according to [13], the standard curve was plotted between the bovine serum albumin concentration verses the absorbance of BSA at 595 nm

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Bacillus cereus*

The percentages of the contamination of bovine milk with *Bacillus cereus* that observed in this study was 15 (15%) from (100) collecting milk sample from different locations in Baghdad/Iraq.

The colony morphology on blood agar at 37°C*, B.cereus* are large, flat or, with smooth colonies surrounded by a wide zone of beta-hemolysis framing the centrally situated colony with a slight green tinge and about 2-5mm in diameter.

While the colony morphology on selective media, Mannitol egg-yolk-polymyxin (MYP) was still a better and standard medium for easy colony differentiation.

This media based on the diagnostic features of *B. cereus* of lecithin hydrolysis and inability to ferment Mannitol, *B. cereus* forms rough pink colonies about (5 mm) in diameter surrounded by a halo of egg-yolk precipitation of lecithin hydrolysis [14].

3.2 Microscopic examination

Bacillus cereus isolates were positive to gram stain with violet colour, rod-shaped 1 µm wide, 5-10 µm long, with circular or square ends arranged singly, pairs or in short chain bacilli.

When the colonies stained with spore dye, the bacilli stained with two stains as, the vegetative cells stained with red colour while the spores were green, oval with central location.

3.3 Biochemical Identification results for *B.cereus*

The 15 isolates showed negative resultes for oxidase, indole, Methyl Red (MR), Gelatin Hydrolysis and urease tests, while gave a positive resultes to the Catalase, Citrate Utilization, VP (Voges Proskauer), Motility, and gave strong +β hemolysis and Starch hydrolysis with positive lecithinase activity.

3.4 API 20E and Vitek2 Systems results for diagnosis *B.cereus*

The (15) isolates that identified as positive with the biochemical tests were subjected to API 20E and vitek 2 system to confirm the diagnosis of biochemical tests.

The results of API20 E system were read after incubation of stripes at 37˚C for 24hrs, the results were record depending on the reading table reaction and compared with standard of the manufacturer, the arginine dihydrolase (ADH) test was positive, gelatinase test (GEL) positive, the tryptophane deaminase (TDA) positive and the Citrate utilization (CIT) positive result while the other tests were negative.

VITEK2 BCL card provides a major advance with highly sensitive method for reliable identification of *B. cereus* in comparison with other phenotypic methods [15]. The results of fifteen isolates of *B.cereus* were identified with Vitek2 system as positive isolates and the probability of the positive results of biochemical test of vitek2 system were between 90%- 94 %.

3.5 Result of Molecular detection of *Bacillus cereus* with PCR

Polymerase Chain Reaction (PCR) has become one of the most important molecular diagnostic methods for detection of pathogens and is considered to be a valuable alternative to the culture-based detection techniques due to its speed, limit of detection, sensitivity and specificity [16].

All the DNA of 15 *B.cereus* isolates were successfully extracted and the concentration of DNA was determined by Nanodrop 1000 spectrophotometer at 260/280 nm, the purity of extracted DNA range from 1.7-2. The extracted DNA was visualized under UV after electrophoresis with 1% agarose gel at 70 volt for 30 min.

Specific primers were used to determine the 16SrRNA genes in this study; the optimal conditions were identified after several experiments these conditions it has been found that the best volume of the DNA template was 5 µl, and the final volume of reaction mixture was 20µl that transferred to a thermal cycler and the denaturation and annealing temperatures were (94˚C, 56˚C) respectively, PCR product appeared as a DNA band with about (676) bp.

The positive result was confirmed by agarose gel electrophoresis in a 2% agarose stained with red safe stain, electrophoresed in 70 volt for 1:30 hr and the 15 lanes were photographed under ultraviolet (UV) transilluminator with (676) bp band size and (100) bp as DNA ladder as in Figure (1).

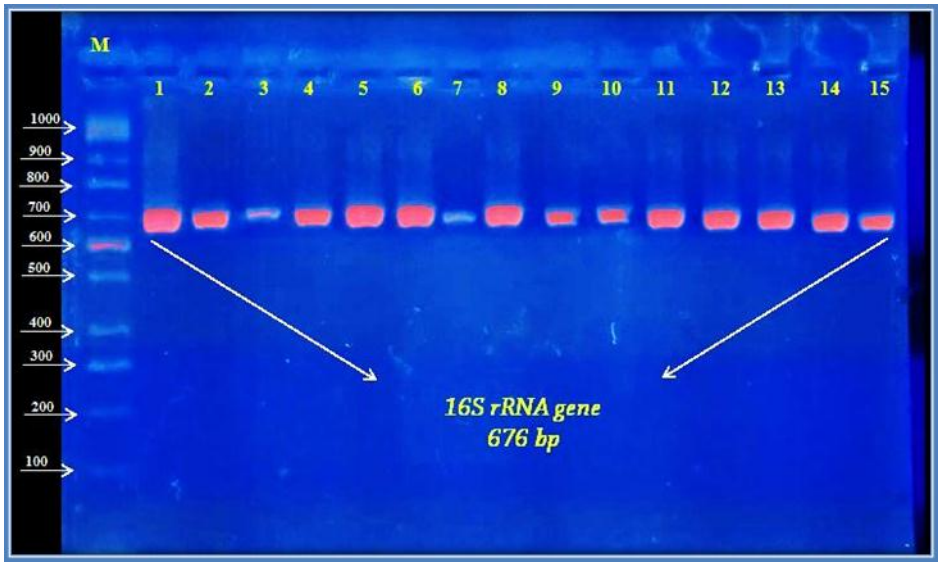

Figure 1: PCR amplification of 16S rRNA was electrophoresis on 2% agarose at 70 volt, 1x TBE buffer for 1:30 hours. M: DNA ladder (100), lanes (1-15), visualized under U.V light, the band size 676 bp.

3.6 The Antimicrobial Activity results of extracted crude *B.cereus* bacteriocin against target Bacteria

Bacillus cereus is an interesting species to investigate for antimicrobial activity since *Bacillus* species produce a large number of peptide antibiotics representing several different basic chemical structures, *B.cereus* produce bacteriocin which is ribosomally synthesized antimicrobial peptide, this bacteriocin, called here cerein [17].

Most of the bacteriocins show a narrow spectrum of action, as they inhibit strains closely related to the producer organisms, while only few bacteriocins inhibit diverse groups of gram-positive and gram negative bacteria.

Cell-free supernatant of 15 isolates of *Bacillus cereus* isolated from bovine milk were tested for the presence of antimicrobial activity against several strains including, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, the isolates designated as (BC1 to BC15), using well diffusion agar method, and the inhibition zone diameter of each isolates against target bacteria was measured, the results shown in table (1) and Figure (2).

The results showed that all the 15 isolates of *B.cereus* have ability to produce bacteriocin with difference in their antimicrobial activity, ranging from large inhibition zone diameter with (15mm) to slight inhibitory effect with (2 mm) while there is no inhibitory effect against some indicator strains.

The isolates of (BC4) shown maximum zone of inhibition and the antimicrobial activity against the indicator strains was (14 mm) for *Staphylococcus aureus*, (8 mm) for *Escherichia coli* and (6 mm) for *Pseudomonas aeruginosa*,

The first study on *B.cereus* bacteriocin reported by *[18]* who revealed that *B.cereus* Strain GN105 that isolated from food produced and secreted a protein factor, called cerein, which strongly reduced the growth of the *B. cereus* indicator strain.

Table 1: The antimicrobial activity of crude bacteriocin of *B.cereus* with inhibition zone diameter of target bacteria.

Diameter of inhibitions Zone per (mm)	*B.cereus* isolates no.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Staphylococcus aureus	13	15	15	14	15	9	12	10	11	11	9	13	8	9	7
Escherichia coli	7	4	5	8	6	7	4	6	2	0	4	5	2	3	0
Pseudomonas aeruginosa	5	6	5	6	2	4	2	4	0	0	0	2	0	0	0

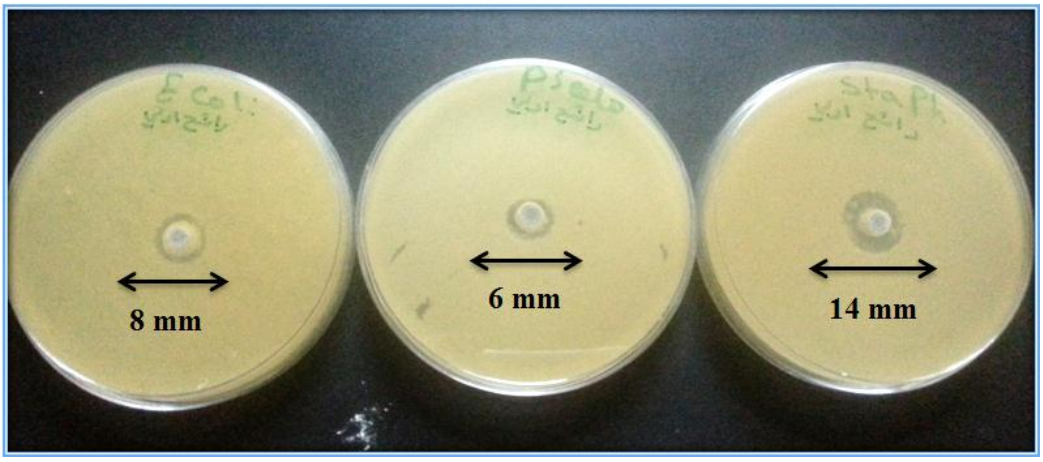

Figure 2: Inhibition zone diameter of crude bacteriocin of *B.cereus* against target bacteria.

3.7 Nucleotide Sequence of 16s ribosomal RNA Gene of *B.cereus*

The selected *B.cereus* (BC4) with forward primer of 16SrRNA was sent to Korea to identify the sequences and compared with similar sequences of the 16S rRNA gene of the reference strains of *B.cereus* in GenBank.

Sequencing analysis was performed for PCR product and one isolates (BC4) from (15) *B.cereus* isolates in this study was selected for sequencing and compared with other 16SrRNA genes in the GenBank by using the NCBI Basic Local alignment search tools BLAST-n program, the partial sequence for *B.cereus* strain with range of nucleotide from 68-654 has identity with 100% percentage compering whit other strain in GenBank without mutation and this strain was designated as *Bacillus cereus* ES102 Figure (3), table (2).

However, early studies performed with a limited number of isolates from the *Bacillus cereus* group revealed that the 16S rRNA sequences of species had as high as a 99 to 100% similarity, another advantage of using the rRNAs as a target is the fact that these molecules are naturally amplified within the cell. In general, rRNA represents about 80% of total nucleic acids in microbial cells and, thus, is present in many hundreds of thousands of copies per cell. This natural amplification allows for direct detection of rRNA sequences.

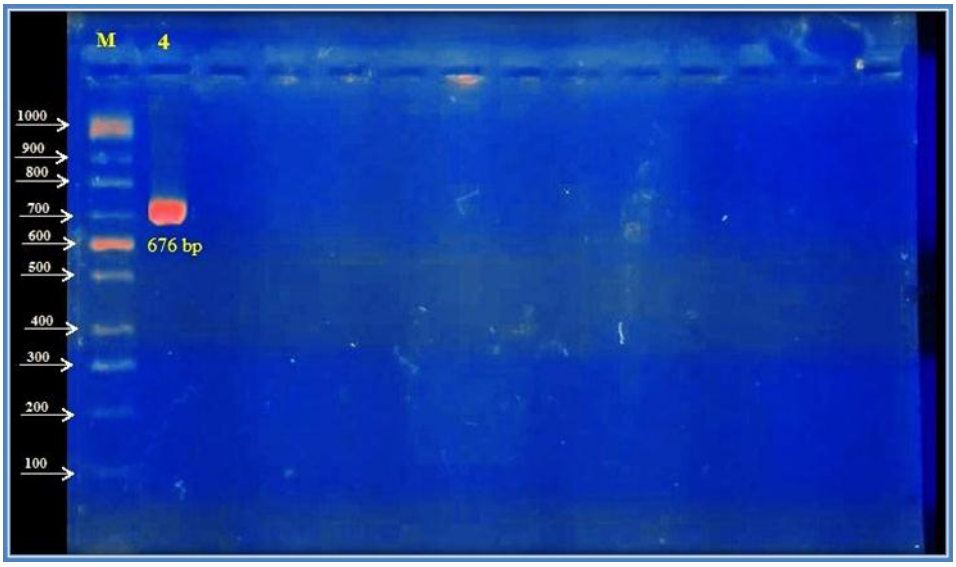

Figure 3: the selected strain of *B.cereus* (BC4) was PCR amplification of 16S rRNA was electrophoresis on 2% agarose at 70 volt, 1x TBE buffer for 1:30 hours. M: DNA ladder (100), lanes (1-15), visualized under U.V light, the band size 676 bp.

Table 2: Partial sequence of 16S ribosomal RNA gene for *Bacillus cereus*.

No. of sample	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID	Score	Expect	Identities	Source
1		----------		68 to 654	ID: KY689039.1	1085	0.0	100%	*Bacillus cereus*

***Bacillus cereus* strain ES102 16S ribosomal RNA gene, partial sequence:**

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68   TAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACC 127
128  GGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGG 187
188  ATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGT 247
248  AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG 307
308  GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA 367
368  GTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAA 427
428  TAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC 487
488  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGT 547
548  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG 607
608  GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG  654
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3.8 Determination of Protein concentration

The result of protein concentration was determined to the extracted crude bacteriocin in this study according to Bradford's method the protein concentration was 0.43 mg/ml.

4. CONCLUSION

The Iraqi isolate of *Bacillus cereus* have ability to produce bacteriocin with antimicrobial activity against gram positive and gram negative bacteria with protein concentration of 0.43 mg/ml, and the isolates identified at molecular sequences and design as *Bacillus cereus* BC4 ES102 which is considered as a novel Iraqi bacteriocin.

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