Bacillus cereus in Meat Products: *16S rRNA* Phylogenetic Tree analysis and Antimicrobial Investigation of Nisin A, Rosemary Essential Oil and Tetracycline

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Abstract

In view of the importance of human food poisoning, this study was aimed to isolate *B. cereus* from meat sources that is economically problem affects both manufacturers and consumers. *16S rRNA* was used to identify bacteria as well as for constructing phylogenetic tree. The activity of different antimicrobial agents (nicin A, Rosemary essential oil and tetracycline) were determined using well diffusion method. *16S rRNA* analysis was successfully identified the three studied isolates from meat as *B. cereus* as well as the phylogenetic tree results observed that bacteria source in meat might be the soil. Rosemary essential oil at concentration 2000 µg/ml was more effectively then nisin A at 32 mg/ml. In conclusions, the *16S rRNA* sequences analysis was efficient for identification of *B. cereus*. Also, the higher antibacterial effect of Rosemary essential oil against *B. cereus* need further studies to be used as preservative agent for meat products.

Key words: *food poisoning bacteriocins, Rosmarinus officinalis, Bacillus cereus*

Introduction

Various kinds of microorganisms can cause food spoilage that is economically problem affects both manufacturers and consumers. *Bacillus cereus* is representing a common food contaminant, and is cause distinct forms of illness such as emetic and diarrheal. *B. cereus* is existing in various foods like meats, milk, vegetables. The counts of *B. cereus* in meat products were 2.9-4.59 Log CFU/g and it can grow well after cooking [(1)]. Nevertheless, some pathogens including the spore forming bacteria may appear lower infectious doses, foodborne disease related to jerky products have been increased. Several methods have been studied for microbial safety of jerky products such as additives to food, heating, and irradiation antagonistic toward pathogens like *Staphylococcus aureus*, *B. cereus, Listeria monocytogenes Escherichia coli* and *Salmonella Typhimurium*, but not handle the quality of jerky products during storage [(2)].

B. cereus sensu stricto known as a food poisoning associated pathogen with sometimes causes in humans soft tissue infections [(3)]. *B. cereus* is vastly distributed in the environment and have a relative genetic relationships to other species in the *B. cereus* group inclusive *B. thuringiensis*, and *B. mycoides* [(4)].

Identification of bacterial species by molecular methods is widely used. The *16S rRNA* gene is a powerful molecular tool which has been proven to be identified the species of bacteria [(5)]. Traditional methods used to detect *B. cereus*, such as selective media and biochemical tests as well as confirming the presence of protein toxin crystals, are time consuming. Molecular methods such as PCR are rapid for the identification these bacteria and many genes used for this purpose

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including *16S rRNA*, *23S rRNA*, *gyrB,* or *16S–23S rRNA* spacer regions [6,7].

Synthetic antimicrobial preservatives were used for many years in the food industry, but their consumption can lead to intoxications, allergies, cancer …etc. Thus, the search for natural products as new antimicrobial agents derived from animals, plants and microorganisms used in food industry [8].

Bacteriocins are divided into two classes: class I and II groups. The most studied one is lantibiotics subclass of bacteriocins, which inclusive nisin, staphylococcin C55, lacticin and mersacidin. Some of lantibiotics have potent activity against food-borne pathogens [9,10].

Plant essential oils have antimicrobial activities; however they limited used in foods due to their strong flavor. Lately, plant extracts as food antioxidant have been developed such as Rosemary extract that tested versus some food spoilage and foodborne pathogenic microorganisms under various conditions of pH, water activity, and temperature [11].

The meat products are widely consumed by the Iraqi people who give concerns about food poisoning caused by *B. cereus* pathogen. Thus, this study was focused on to isolate *B. cereus* from meat sources that deals with human food poisoning and to identify this microorganism used biochemical and molecular techniques as well as *16S rRNA* for constructing phylogenetic tree. Furthermore, compare between the activity of nicin, rosemary essential oil and tetracycline as antibacterial and food preservative material.

Materials and Methods

Isolates and culture media

One hundred food samples were collected from Baghdad markets from July 2016 to September 2016, including Fried meat, Grilled meat, and Beef burger buy from local market. All samples were primary cultured on serial dilutions were prepared, and 0.1 ml of each diluted sample was streaked in mannitol yolk polymyxin agar (MYP) a medium used for the enumeration, detection and isolation of *B. cereus* in food samples were incubated for 24 h at 30°C. The colonies (peacock blue) with lecithinase zone were transferred it on nutrient agar for further study [12].

Molecular assay

Extraction of DNA and polymerase chain reaction

The DNA of bacterial isolates was extracted based on the protocol of Wizard Genomic DNA Purification Kit, Promega. Concentration of DNA was measured by Quantus Florometer. Amplification of *16S rRNA* was done using the primers 27F (AGAGTTTGATCTTGGCTCAG) and 1492R (TACGGTTACCTTGTTACGACTT) (13). The PCR mixture was consisted of 12.5µl of GoTag Green Master Mix (Promega, USA), one µM of above primers and 2 ng/µl of tested DNA completed to 25 µl with nuclease free water. PCR protocol was conducted with an initial denaturation for 5 min at 95ºC; 30 cycles of denaturation for 30 sec at 95ºC, annealing for 45 sec at 60ºC, extension for 1min at 72ºC then final extension for 7 min at 72ºC. After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. Under UV transilluminator visulazation, the PCR product was separated in 1% agarose gel to determine the size of amplicons.

Sequencing

PCR amplicons were sending for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation, Korea. The sequences of 16S rRNA were built up from forward and reverse sequence data using Genious software. DNA sequencing data was analysed via Basic Local Alignment Search Tool (BLAST) of NCBI GenBank (http:// www.ncbi.nlm.nih.gov). MEGA7 sequence analyzing software with 500 bootstrap values was used for constructing the phylogenetic tree.

Antibiotic susceptibility test

All presumptive *B. cereus* isolates were confirmed by AST. The cultures were grow on Muller Hinton Agar plates and incubated at 37°C for 18 hrs with the following antibiotics: Penicillin (10µg⁄ Disc), Amoxicillin (25µg⁄ Disc), Ampicillin (10µg⁄ Disc), Ceftriaxone (5µg⁄ Disc), Cloxcillin (30µg⁄ Disc), Tetracycline (30µg⁄ Disc).The inhibition zone around disk were recorded and compared with Clinical and Laboratory Standards Institute [14].

Preparation of Nisin A

Nisin A (Sigma Nisaplin 2/5%, Germany) was prepared through dissolved with 100mg from Nisin A powder by 10 ml HCl (0.02 N) to give10^4 IU/ml (40 IU=1 g) concentration. After that the solution was passed throughout 0.45 filters for sterilization then stored at -20 ºC [15].

Preparation of essential oil of *R. officinalis*

The essential oil of Rosemary was extracted from leaves dried by air (250g) using Cleavenger hydrodistillation method. The distilled water with plant material was boiled for 3h, the essential oil was stored at 4ºC until use [16].

Prepared Tetracycline powder stock solution:

Described by Andrews (17): The (Tetracycline) stock suspension (50 mg/ml) was performed via solute 0.5g from vancomycin powder in 10 ml D.W.

Determination of the inhibitory effect of antimicrobial agents

Determination the antimicrobial effect of the nisin A, Rosemary essential oil and tetracycline on *B. cereus* planktonic cell using well diffusion assay method was applied according to [18].

Statistical Analysis

The data were analyzed using the SPSS IBM version 20. Least significant differences (LSD) test was done to investigate the differences between diameters of inhibitory zone of tested antimicrobial agent. Values were considered statistically significant $P \leq 0.05$.

Results and Discussion

Meat has exerted a crucial role in human evolution and is an important component of a healthy and well balanced diet due to its nutritional richness and importance in human nutrition as well as examine some pejorative beliefs about meat consumption [19].

The PCR results for four studied bacterial isolates were analyzed using gel electrophoresis that showed the amplicons size of *16S rRNA* gene was 1500 bp in Figure 1.

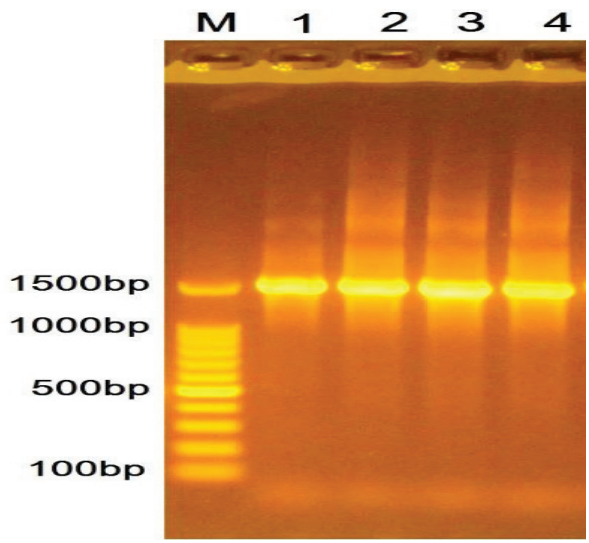

Figure 1. PCR product of the *16S rRNA* gene (1500 bp) from bacterial DNA stained with ethidium bromide. Lane M. 100 bp DNA ladder, Lanes 1-4. Unknown bacterial isolates. The product was electrophoresis on 1% agarose at 5 volt/cm2. 1x TBE buffer for 90 min.

The forward sequences of three PCR products of the *16S rRNA* gene were compared with the GenBank database using BLAST in NCBI website to find the related sequences. The three studied sequences showed high similarity to the sequences registered found in GenBank as illustrated in Table 1. BCM-1, BCM-2 and BCM-3 observed 99% identity to *B. cereus* ATCC 14579.

Among large numbers of genes within a bacterial genome, the *16S rRNA* gene play as an initial key for phylogeny based identification when compared against *16S rRNA* gene sequence GenBank databases [20]. The taxonomists concluded that 70% or higher DNA-DNA relatedness with 5% or less divergence within related sequences, together with 97% or higher *16S rRNA* sequence similarity, is the best means of defining a species [6].

Table 1. Bacteria isolated from meat identified according to the results of a BLAST on the GenBank database in NCBI.

Isolates	Accession number in GenBank	Closest species in GenBank database	Similarity Index			
			Score(bit)	E-value	Identity	Gap
BCM-1	NR_074540.1	Bacillus cereus ATCC 14579	2540	0.0	99%	0%
BCM-2	NR_074540.1	Bacillus cereus ATCC 14579	2582	0.0	99%	0%
BCM-3	NR_074540.1	Bacillus cereus ATCC 14579	2560	0.0	99%	0%

The phylogenetic results of *16S rRNA* for studied isolates from meat and strains from GenBank database showed that the first two isolates (BCM-1 and BCM-2) arranged together in a sister group but BCM-3 isolate divergent from them. All studied isolates in phylogram belong to *B. cereus* bacteria as well as showed direct lineage with *B. thuringiensis*. The *B. cereus* group has several *Bacillus* species that are related physiologically and have a significant genetic similarity to *B. cereus* bacteria including *B.cereus*, *B. anthracis, B. thuringiensis* and *B. mycoides* [14].

This gene is used in phylogenetic building and taxonomy due to it is universal found in different bacterial species. Because of rapid evolutionary rate that contributes to lower the percentage of gene similarities resulting in *16S rRNA* is appropriate for bacterial identification

till closely related species. Results of Bavykin *et al.* [6] showed that closely related species might be reach to 100% *16S rRNA* gene similarity which makes it difficult to distinguish between them. Whereas Wang *et al.* results observed the gene similarity reach to 99.2% when used *gyrB* [21]. Phylogenetic tree of the three studied isolates showed closer to *B. cereus* isolated from soil which suggest that the source of *B. cereus* in meat may be soil.

The results of disk diffusion method (Figure 2) showed various levels of susceptibilities to different antibiotics among studied isolates. The isolates of *B. cereus* observed as multi-resistance for used antibiotics especially against Amoxicillin and Cloxcillin while sensitive to Tetracycline that were similar to results obtained by [22].

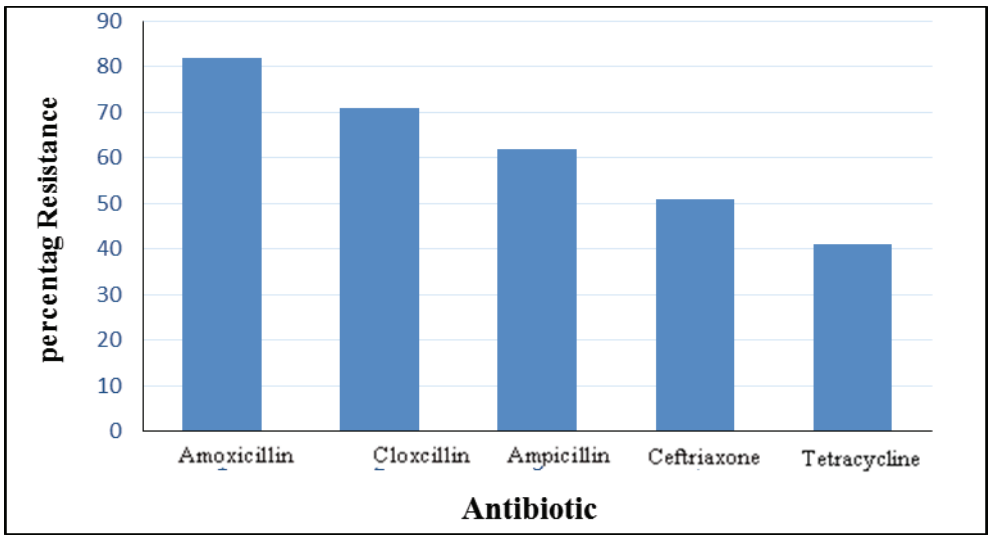

Figure 2. Antibiotic susceptibility of *B.cereus* isolates against group of antibiotics.

As shown in Figure 3, the antimicrobial agents used in this study showed highly significant differences (P≤ 0.01) in inhibitory zone diameters among them and among different concentrations of each agent except for the first concentration of niacin and tetracycline was not significant(P>0.05).

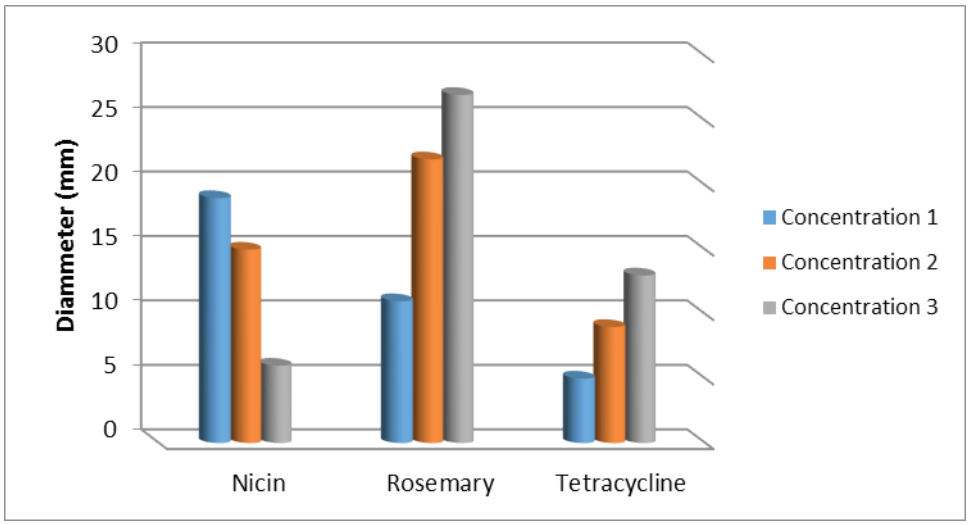

Figure 3: Inhibitory zone diameter of nicin A, Rosemary essential oil and Tetracycline at different concentrations against *B. cereus* bacteria

The nisin A inhibitory effect at concentrations 32 and 64 µg\ ml were measured as diameter that shown in figure 4 while at 128µg\ ml lower diameter. The result agreed with [22] the antimicrobial effect of nisin was investigated against *B. cereus* at different concentration during storage.

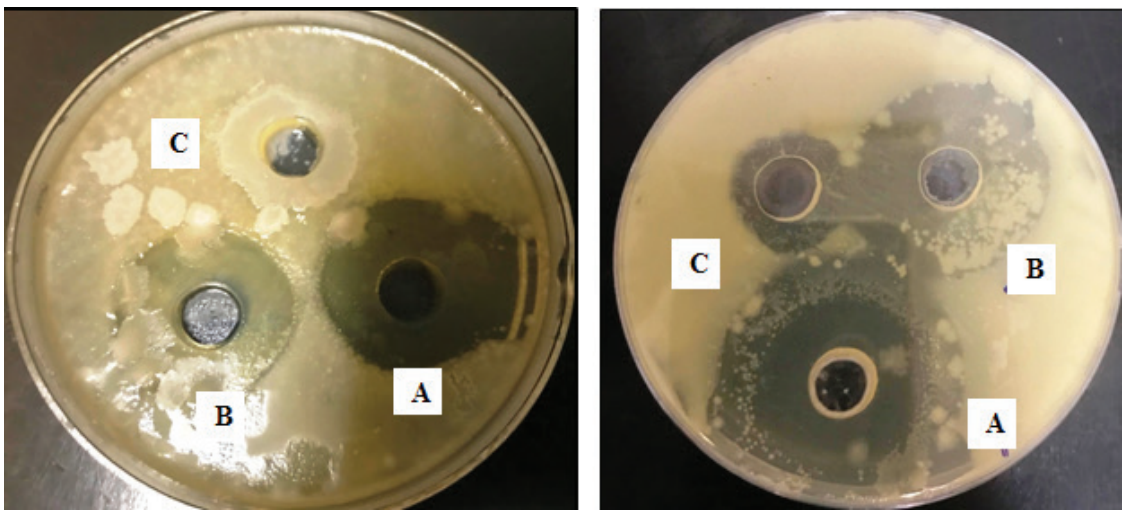

Figure 4: MIC for nicin A at concentrations (A) 32, (B) 64 and (C) 128 µg\ ml against *B. cereus* on Muller Hinton agar at 37°C for 24 hr.

The three concentrations (500,1000 and 2000 µg/ ml) of Rosemary essential oil against *B. cereus* observed diameter of inhibition zone reached to 11, 22 and 27 mm respectively (Figure 5). The result agreed with [23] which reported the rosemary extract abuse commercially in food as an antioxidant of lipids which was examined against foodborne microorganisms.

Natural herbal products and their derived compounds have used in many studies for controlling the growth of pathogens in food products. In investigation studies on *B. cereus* found that Rosemary essential oil have broad spectrum of antibacterial activity against this bacteria [24]. Combination of thyme oil (0.4%) with cinnamon oils (0.4%) decreased the count of *B. cereus* growth in

minced meat to reduction percentage 96.09%. Moreover, the shelf-life of mortadella has been extended with the use of rosemary/thyme EOs [25].

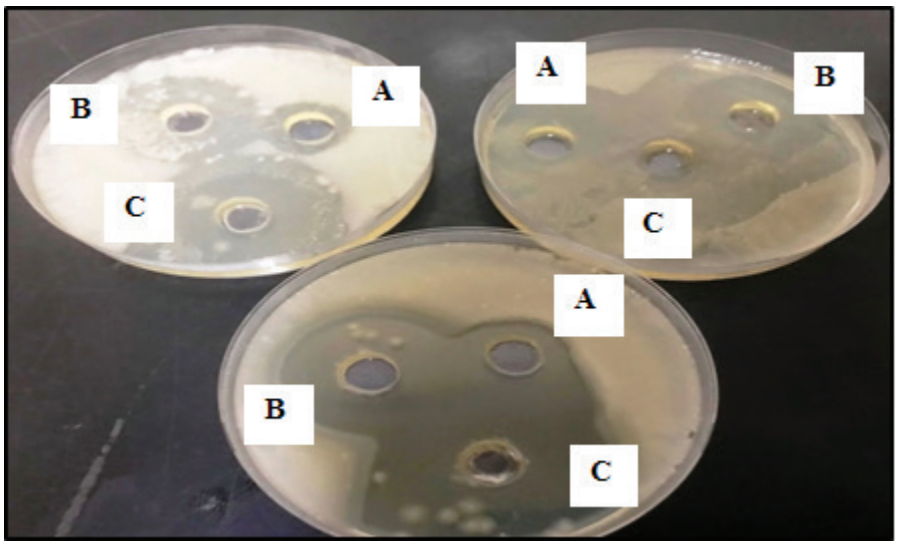

Figure 5: MIC for Rosemary essential oil at concentrations A) 500, B) 1000 and C) 2000 against *B. cereus* on Muller Hinton agar at 37°C for 24 hr.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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