African Journal of Advanced Pure and Applied Sciences (AJAPAS)
Online ISSN: 2957-644X
Volume 3, Issue 2, April - June 2024, Page No: 180-187
Website: https://aaasjournals.com/index.php/ajapas/index
معامل التأثير العربي 2023: (1.55) **SJIFactor 2023: 5.689** **ISI 2022-2023: 0.557**

Isolation of *Bacillus Cereus* from Meat Ready to Eat Using Antimicrobial Resistance Pattern and Polymerase Chain Reaction - Deoxyribonucleic Acid (DNA-PCR)

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Received: April 05, 2024	Accepted: June 10, 2024	Published: June 24, 2024

Abstract:
A total number of 50 of ready to eat mutton meat samples were purchased from 10 different restaurants at Tripoli Libya on September 2022. Mannitol egg yolk polymyxin (MYP) media with polymyxin *B. cereus* was used to enumerate and isolate *Bacillus cereus* from (RTE) meat. twelve isolates were biochemically identified as *B. cereus*. All isolates were positive toward lecithinase production, starch hydrolysis, nitrate reduction, catalase production, Gram stain, glucose fermentation. None of the samples were positive towards indole, sucrose, H2S, gas production and lactose fermentation. The *B. cereus* isolates were examined for DNA-polymerase chain reaction (DAN-PCR) using primer S30 (5'-GTGATCGCAG-3') Antimicrobial pattern illustrated four pattern and phenotypically less heterogeneous when compared to DNA-PCR. A total number of nine types of *B. cereus* have been produced by a combination of phenotype and genotype methods. These results demonstrated that both typing method provides evidence toward the existence of relatedness and diversity of the *B. cereus* strains from RTE mutton meat.

Keywords: *Bacillus Cereus*, Antimicrobial, RTE, Deoxyribonucleic, PCR, Isolation - MYP.

Cite this article as: A. A. M. Ali, "Isolation of Bacillus Cereus from Meat Ready to Eat Using Antimicrobial Resistance Pattern and Polymerase Chain Reaction - Deoxyribonucleic Acid (DNA-PCR)," *African Journal of Advanced Pure and Applied Sciences (AJAPAS)*, vol. 3, no. 2, pp. 180–187, April-June 2024.

عزل بكتيريا *Bacillus cereus* من اللحوم الجاهزة للأكل باستخدام نمط مقاومة مضادات الميكروبات وتفاعل البوليميراز المتسلسل ـ حمض الديوكسي ريبونوكلييك (DNA-PCR)

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الملخص
تم شراء إجمالي 50 عينة من لحم الضأن الجاهز للأكل من 10 مطاعم مختلفة في طرابلس ليبيا في سبتمبر 2022. تم استخدام وسط صفار بيض المانيتول بوليميكسين (MYP) مع بوليميكسين B. cereus لتعداد وعزل Bacillus cereus من (RTE) لحمة. تم تشخيص اثنتي عشرة عزلة كيميائيا حيوياً على أنها B. cereus. كانت جميع العزلات إيجابية تجاه إنتاج الليسيثيناز، تحلل مائي للنشا، اختزال النترات، إنتاج الكاتلاز، صبغة جرام، تخمر الجلوكوز. لم تكن أي من العينات إيجابية تجاه الإندول والسكروز وكبريتيد الهيدروجين وإنتاج الغاز وتخمير اللاكتوز. تم فحص عزلات B. cereus من أجل تفاعل البلمرة المتسلسل DNA (DAN-PCR) باستخدام البادئ S30 (5'-GTGATCGCAG-3') وهو نمط مضاد للميكروبات موضح بأربعة أنماط وأقل تجانساً ظاهرياً عند

مقارنتها بـ DNA-PCR. تم إنتاج إجمالي تسعة أنواع من B. cereus من خلال مزيج من طرق النمط الظاهري والنمط الجيني. أظهرت هذه النتائج أن كلتا طريقتي الكتابة توفر دليلاً على وجود الارتباط والتنوع بين سلالات B. cereus من لحم الضأن RTE.

الكلمات المفتاحية: البكتيريا العصوية الشمعية، مضادات الميكروبات، الديوكسيريبونوكلييك، PCR، العزلة.

Introduction

B. cereus is recognized as a foodborne pathogen and is a spoilage microorganism that has been associated with the development of quality defects. This microorganism is recognized as a foodborne pathogen and is capable of causing two types of foodborne illness vomiting and diarrhea syndromes. Symptoms are generally mild and short-lived (up to 24 hours*). B. cereus* is commonly found in the environment (e.g. soil) as well as a variety of foods. Spores are able to survive harsh environments including normal cooking temperatures [10], [12] Meat is a nutritious, protein-rich food which is highly perishable and has a short shelf-life unless preservation methods are used [5] Foodborne diseases are an increasingly recognized problem involving a wide spectrum of illnesses caused by bacterial, viral, parasitic or chemical contamination of food. Although viruses account for half of all the foodborne illnesses, most hospitalizations and deaths related to foodborne infections are due to bacterial agents. Diarrheal diseases are the commonest manifestation of food poisoning and in some cases, can lead to death. The diseases are caused by either toxin from the "disease-causing" microbe, or by the human body's reactions to the microbe itself [12] Endospore-forming bacteria such as *B. cereus* represent one of the highest risks and capacities to produce food-poisoning toxins in low-acid foods due to heat resistance of endospores and the broad temperature range in which some strains can grow. Ready-to-eat (RTE) meat has been receiving popularity among Libyan as their lunch and dinner. Yet there are still needs to evaluate the food safety of the product in Libya. To date, there is no official information is available on the incidence of *B. cereus* poisoning in Libya. Therefore, the present study was carried out to assess the microbiological quality of RTE meat foods supplied in Tripoli, Libya in a bid to throw more light on the inherent risk associated with such foods.

Material and methods

Sample Collection a total number of 50 mutton meat samples were purchased from 10 different restaurants at Tripoli Table 1. All samples were collected after 6 hours of cooking, and were placed in clean dry sterile bags and transported in icebox to the laboratory for bacteriological analysis.

Sample Preparation the mutton meat sample was aseptically cut into smaller pieces using a sterile knife. Twenty-five grams of the food samples were homogenized by a stomacher in 225 ml of peptone water for 1 minute. Several dilutions were achieved (1×10-1 to 1×10-5) up to five-fold for each prepared by transferring 1 ml from stock homogenate to 9 ml of sterile peptone water, mixing well with vigorous shaking and continuing until 10-5 dilution is reached. This was carried out in order to obtain separated colony [8], [1].

Table 1 Samples collection of meat.

Region	Restaurant
Tripoli	Multiple restaurants
Total	**50**

Plating of presumptive *Bacillus cereus*

Spread-plate procedure was carried out as follows: 0.1 ml volume of the 1×10-5 dilutions was spread evenly onto surface of each plate with sterile glass spreading rod in a selective media mannitol-egg yolk-polymyxin (MYP) agar (Oxoid), and incubated at 30°C for 24 hours. The total counts of B. cereus are based on percentage of colonies tested that were presumptively positive toward *B. cereus*, and expressed as cfu/g (cfu stands for colony-forming unit. This means that cfu/g is colony-forming unit per gram and cfu/ml is colony-forming unitper millilitre. A colony-forming unit is where a colony of microbes grow on a petri dish, from one single microbe, each one of these would be a colony-forming unit.) so cfu/g of sample as follows $N = C / V(n) \times D$, where C is the sum of colonies on all plates count, V is the volume applied to each plate, n is the number of plates and D is the dilution from count obtained. four or more colonies of presumptive B. cereus were randomly selected from MYP agar and subcultured onto nutrient agar slant (Oxoid) and incubate for 18—24 hours at 30°C. The samples were stored at 4°C until use for identification and confirmation [8].

Identifection and confirmation

Of *Bacillus cereus* Characterization and identification of the samples were achieved by initial morphological examination of the colonies in the plate for colonial appearance and lecithinase production results were recorded. Further identification of the presumptive *B. cereus* colonies using biochemical characteristics including Gram stains, catalase production test, nitrate reduction test, glucose fermentation test, lactose fermentation test,

Hydrogen sulfide (H2S) production test, gas production test, motility test and indole production test. Physiochemical identification were carried out as described by [4] , [13].

Antibiotic sensitivity test

Antimicrobial susceptibility was determined by the disc diffusion agar method (CLSI 2006). Single colonies of 24 hours-old cultures were transferred to 5 ml of tryptic soy broth (TSB) (Difco) and incubated at 37°C for 2-6 hours. A sterile cotton swab was dipped into the TSB growth was applied evenly onto pre-dried Mueller-Hinton agar (Difco) plate. After drying for 15 minutes, the antimicrobial test discs namely Ceftriaxone 30 µg, Streptomycin 25 µg, Chloramphenicol 30 µg, Ciprofloxacin 5 µg, Vancomycin 30 µg, Clindamycin 2 µg and Nalidixic acid 30 µg (Himedia) were placed aseptically and the plates were incubated at 37°C for 14-19 hours. The zones were measured as follow, ≥15 sensitive and ≤14 resistance according to the standard methods [3], [2].

DNA extraction

DNA extraction was done using boiling method [9]. and Promega wizard genomic DNA purification kit [7]

Boiling method

the cells were grown in brain heart infusion broth (Sigma) at 30 °C for 18-24 hours were harvested and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded. The pellet was then washed with 1.0 ml sterile distilled water and vortex. Then, it was boiled at 98°C for 10 minutes and immediately was kept frozen at -20°C for 10 min, this step was repeated twice. Then the tube was centrifuged at 10,000 rpm for 5 minutes. The supernatant was used as a template [9].

Extraction using DNA purification

one ml of overnight culture was centrifuged for 2 minutes at 13,000–16,000 rpm. The supernatant was discarded. The cells were suspended in 480 µl 50mM EDTA. Lytic enzymes (120 µl) (lysozyme and/or lysostaphin) were added and incubated at 37°C for 30–60 minutes. The cells were centrifuged for 2 minutes at 13,000–16,000 rpm and the supernatant were removed. A total of 600 µl Nuclei Lysis Solution was added to the tube and incubated for 5 minutes at 80°C, then cooled to room temperature. A total of 3 µl of RNase Solution was then added and incubated at 37°C for 15–60 minutes, then cooled to room temperature. A total of 200 µl of protein precipitation solution was added, vortex and incubated on ice for 5 minutes, then centrifuged at 13,000–16,000 rpm for 3 minutes. The supernatant was transferred to a clean tube containing 600 µl of room temperature isopropanol, mix and centrifuged for 2 minutes at 13,000–16,000 rpm. The supernatant was discarded and 600 µl of room temperature 70% ethanol was added, mix and centrifuged for 2 minutes at 13,000–16,000 rpm, then air-dry the pellet for 10–15 minutes and rehydrated the DNA pellet in 100 µl of Rehydration Solution for 1 hour at 65°C as it described in the technical manual [7].

Qualitative and quantitative of extracted DNA

A total of 2 µL volumes were needed to quantify DNA, the concentration measurement was performed using MaestroNano Spectrophotometer by taking the ratio of absorbance 260 nm over the absorbance at 280 nm. the DNA concentration represent the quantity in ng/ul.

Primer

The random primer has the 50% G+C content gene sequence (10-mer) was screened and primer, the S30 (5'-GTGATCGCAG-3') (FirstBase Malaysia) was selected for the study as it provides reproducible and discriminatory pattern [6], [14]

PCR amplification

the RAPD-PCR fingerprinting assay was performed in a 25 µl volume each tube contained GoTaq green master mix (FirstBase Malaysia) (2.5 µl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.1) and 0.1% Triton™X-100), 1.5 µl 50 mM MgCl 2, 0.5 µl of 10 mM dNTPs, 0.4 µl of 5 units of Taq DNA polymerase) and 1.0 µl of 100 mM primer (S30), 6.5 µl water nuclease-free and 5 µl of 10 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water [9] Amplification was performed in Eppendorf Master-cycler nexus GSX1 (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94 °C for 5 minutes followed by 40 cycles of denaturation at 94 °C for 15 second, annealing for 15 second at 36.5 °C and polymerization at 72 °C for 2 minutes. Final elongation was at 72 °C for 4 minutes [6],[15].

Separating DNA fragments by agarose gel electrophoresis

the amplification products were analyzed by electrophoresis in a 2% agarose in 1.0 X TAE (0.1 M Tris, 0.1 M Boric acid, 0.1 mM EDTA) at 100 V for 45 minutes [6] Gels were stained with maestro-safe nucleic acid pre-

stained (2 µl in 100 ml TBE). The amplified fragments were visualized digitally by using UV trans illuminator [7]

Results and discussion

Plate count of *Bacillus cereus*

the presumptive *B. cereus* colonies appeared as a dry-rough surface, red-purple in colour and surrounded by white precipitated egg yolk on MYP agar. The use of MYP agar for the detection of *B. cereus* is used to diagnose the non-fermentation of mannitol and lecithinase production. Polymyxin B was included as a selective agent to inhibit Gram negative organisms.

Table 2 shows the total mean count (cfu/g) and the standard deviation of presumptive *B. cereus* for all samples that were collected from 10 different restaurants at Tripoli in statistic for compare was used to interpret the result based on 95% confidence.

samples has scored the highest mean of the presumptive *B. cereus* at $1.55\times10^8\pm26.20$ cfu/g, $1.44\times10^8\pm73.61$ cfu/g, $1.33\times10^8 \pm 61.37$ cfu/g, $1.22\times10^8\pm26.33$ cfu/g respectively, followed by samples with scored at $6.11\times10^7\pm27.73$ cfu/g, $5.22\times10^7\pm41.88$ cfu/g, $4.22\times10^7 \pm33.8$ cfu/g, $3.1\times10^7\pm11.44$ cfu/g respectively samples have the lowest means at $2.77\times10^7\pm3.88$ cfu/g, $2.66\times10^7\pm6.21$ cfu/g, $2.33\times10^7\pm3.22$ cfu/g, $2.63\times10^7\pm4.88$ cfu/g respectively.

Table 2 Total mean cfu/g and standard deviation of presumptive *Bacillus cereus*.

Region	B. cereus
Different restaurants at Tripoli	$1.55\times10^8\pm26.20$ cfu/g
	$1.44\times10^8\pm73.61$ cfu/g
	$1.33\times10^8 \pm 61.37$ cfu/g
	$1.22\times10^8\pm26.33$ cfu/g
	$6.11\times10^7\pm27.73$ cfu/g
	$5.22\times10^7\pm41.88$ cfu/g
	$4.22\times10^7 \pm33.8$ cfu/g
	$3.1\times10^7\pm11.44$ cfu/g
	$2.77\times10^7\pm3.88$ cfu/g
	$2.66\times10^7\pm6.21$ cfu/g
	$2.33\times10^7\pm3.22$ cfu/g
	$2.63\times10^7\pm4.88$ cfu/g

No. samples: (cfu/g)
(±); mean and standard deviation

Figure 1 showed significantly high contamination level at 30 °C, which confirms their increased hazard to human. This remarkable rise in bacterial counts in Tripoli could be as a result of poor processing method or poor hygiene practice.

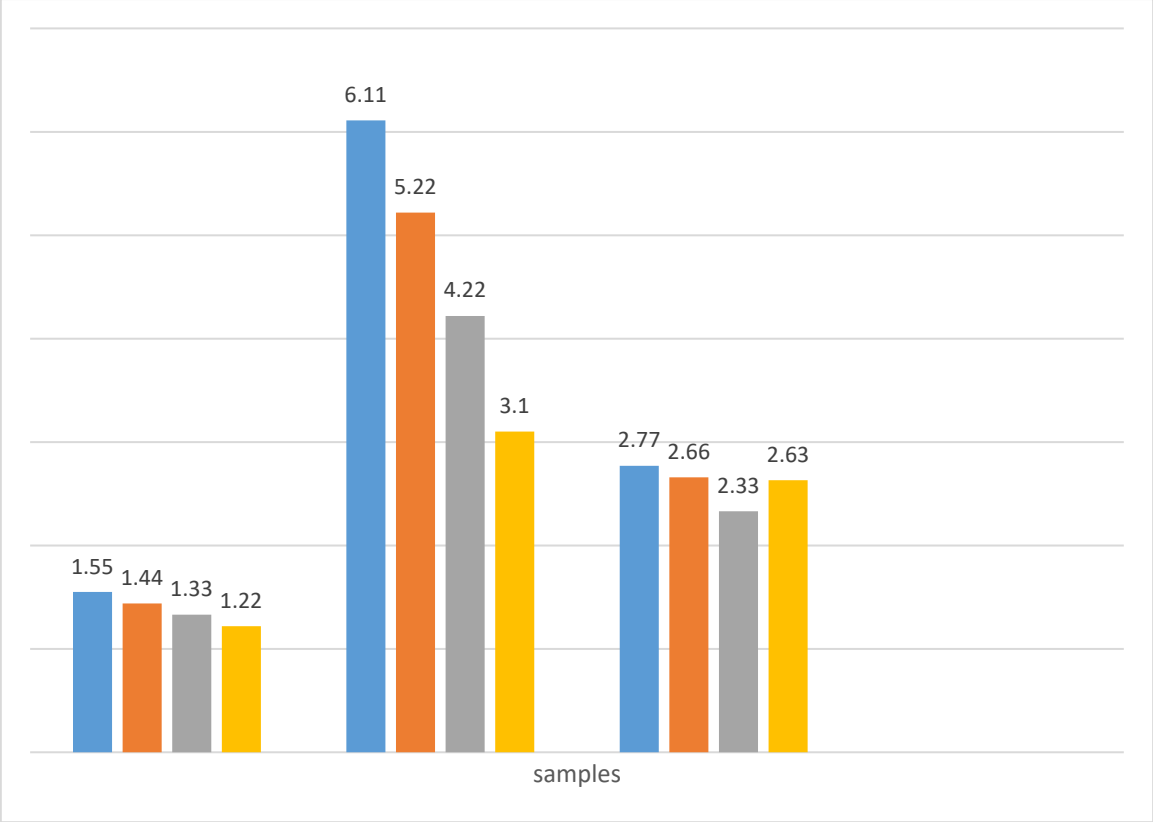

Figure 1 Total mean cfu/g and standard deviation of presumptive *Bacillus cereus*.

Confirmation of *Bacillus cereus*

The biochemical characteristics of presumptive *B. cereus* colonies were determined and compared with those described in BAM (FDA 1992). 12 isolates exhibited uniform phenotypic properties and confirmed as *B. cereus* (Table 3).

Table 3 Isolation and biochemical characterization of *Bacillus cereus*.

Sample	Mo	Lec	Gr	Cat	Ind	Nit	Sta	Glu	Suc	Lac	H2s	Gas	Mt
S1	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S2	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S3	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S4	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S5	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S6	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S7	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S8	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S9	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S10	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S11	Rod	+	+	+	—	+	+	+	—	—	—	—	+
S12	Rod	+	+	+	—	+	+	+	—	—	—	—	+

(Mo); Morphology (Lec); Lecithinase (Gr); Gram-stains (Cat); Catalase (Ind); Indole (Nit); Nitrate reduction (Sta); Starch (Glu); Glucose (Suc); Sucrose (Lac); Lactose (H2S); Hydrogen sulfide production (Gas); Gas production (Mt); motility.

Antimicrobial resistance pattern

Antimicrobial agents have been widely used in human and animal populations to control infectious diseases. *B. cereus* isolates were tested with seven antibiotics by the disc diffusion agar method (CLSI 2006). The zone of inhibition of different antibiotics used and antimicrobial resistance pattern of *B. cereus* isolates were given in Table 4 and 5 respectively.

All *B. cereus* isolates were resistance to ceftriaxone (99 %), vancomycin (86.4%), Clindamycin (90.7%) and nalidixic acid (100%). All *B. cereus* isolates were sensitive to ciprofloxacin (99 %). Also, the majority of the *B. cereus* were sensitive to streptomycin (92.6%) and chloramphenicol (81.4%). The findings of the present study are similar with those obtained by other researchers (Agwa et al. 2012; Guven et al. 2006; Vijaya Kumar et al. 2012). except the samples that were inoculated with vancomycin were surprisingly resistance for those isolated strains. The variations in the percentages may be due to the differences in the concentration of the antimicrobial agents used. Resistance to antibiotics in foodborne pathogens may create problems for disease or illness treatment while antibiotic susceptibility leads to healing of the illness which the organisms caused.

Table 4 Zone of inhibition of different antibiotics used.

Restaurant	Cro	C	Cip	Da	S	Na	Va
S1	R	S	S	R	R	R	R
S2	R	S	S	S	S	R	R
S3	R	S	S	S	S	R	S
S4	R	S	S	R	S	R	R
S5	R	S	S	R	S	R	S
S6	R	S	S	R	R	R	R
S7	R	S	S	R	S	R	R
S8	R	S	S	R	S	R	R
S9	R	S	S	R	S	R	R
S10	R	S	S	R	S	R	R
S11	R	S	S	R	S	R	R
S12	R	S	S	R	S	R	R

R: resistance ≤ 14 mm
S: sensitive ≥ 15 mm
The total number of strains tested were 12 from Tripoli

Tested for Ceftriaxone (Cro); Streptomycin (S); Chloramphenicol (C); Ciprofloxacin (Cip); Vancomycin (Va); Clindamycin (Da); and Nalidixic acid (Na).

Table 5 Antimicrobial resistance pattern of *Bacillus cereus*

Antimicrobial tested	Total no. (%) of strains resistance
Ceftriaxone	99 % (12)
Chloramphenicol	81.4 % (12)
Ciprofloxacin	99 % (12)
Clindamycin	90.7 % (12)
Nalidixic acid	100 % (12)
Streptomycin	92.6 % (12)
Vancomycin	86.4 % (12)

The total number of strains tested were 12 from Tripoli.

Random amplification of polymorphic DNA using PCR

the range of the DNA concentration of all isolates were ≥150 ng/ul provide by Maestronano Spectrophotometer. One primer was selected for RAPD-PCR for twelve isolates of B. cereus namely S30 (5'-GTGATCGCAG-3'). In the present study, 62.5% of these isolates were able to generate a good number of polymorphic bands and the positions of these bands varied between the isolates. 32.5% of the isolates were unable to differentiate the bacterial cultures with the same primer as the DNA-PCR gave very few or no amplified DNA band. However, these weak reactions might be explained by some genetic variation in the target sequence of the primer in these genes. Bands with the same migration distance were considered as a profile. The RAPD-PCR with the primer S30 pattern provides evidence toward the existence of relatedness and diversity of the B. cereus strains.

Previous study used the same primer S30 where all the *B. cereus* strains tested generated two bands, 0.91 and 0.5 kb. The 0.5 kb band is an internal part of ytc P, is a useful marker for bacilli, whereas the 0.91 kb band seems useful as a B. cereus species-specific marker [6]. The RAPD analyses conducted with primer S30 generated nine different profiles namely (P1, P2, P3, P4 and P5) consist numbers of bands that have same molecular weight, whereas antibiotic resistance provide four patterns namely (R1, R2, R3 and R4) whereas R represent a group of antibiotics that are resistance by the strains. The result indicate that the RAPD-PCR was more heterogeneous when compared to antibiotic resistance patterns. With a combination of two different method, nine types of *B. cereus* have been detected in this study (Table 6).

Table 6 Typing among RTE meat isolates of *B. cereus* using antibiotic resistance patterns and RAPD-PCR profiles.

Strains no.	Antibiotic resistance patterns and their groups		RAPD-PCR profiles	Bacillus cereus types
S1	CroCDaNaSVa	R1	N	1
S2	CroNaVa	R2	N	2
S3	CroDaNaVa	R3	P1	3
S4	CroDaNaSVa	R4	P2	4
S5	CroCNaVa	R4	N	5
S6	CroCDaNaSVa	R1	P3	6
S7	CroDaNaVa	R3	P4	7
S8	CroDaNaSVa	R4	P5	8
S9	CroDaNaVa	R3	N	9
S10	CroDaNaVa	R3	N	10
S11	CroDaNaVa	R3	N	11
S12	CroDaNaVa	R3	N	12
Total		**4**	**5**	**9**

R; Group of antibiotics that are resistance by the strains.
P; Number of profiles with same molecular weight
N; No patterns

Conclusion

In this study the *B. cereus* was successfully isolated and identified from mutton meat. All restaurants were found to be contaminated with *B. cereus* at some level. These meat isolates should be regarded as potential toxin producers and therefore cannot explain the low incidence of food poisoning due the consumption of meat containing high numbers of *B. cereus*. The RAPD-PCR with the primer S30 pattern provides five different patterns which is evidence toward the existence of relatedness and diversity of the *B. cereus* strains from mutton meat. It is also noted that the antibiotic resistance pattern was successfully useful for the differentiation of *B. cereus* strains providing four patterns. The results in the presence study demonstrated a combination of phenotypically and genotypically method show nine types of *B. cereus* among RTE mutton meat isolates. The contamination of food may occur at any stage of the process from the slaughter-house to the consumers. B. cereus food poisoning is principally associated with temperature abuse during the storage of cooked foods thus an adequate strategy should be taken to control this pathogen.

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