MOLECULAR DETECTION OF ENTEROTOXIN (*CYT K*) GENE AND ANTIMICROBIAL SUSCEPTIBILITY OF *BACILLUS CEREUS* ISOLATES FROM MILK AND MILK PRODUCTS

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ABSTRACT

Two hundred twenty seven samples of milk, soft cheese, curls cheese and yogurt were taken to isolate *B. cereus*. The percentage of these bacteria was 32.7 %, 16.6 %, 18 % and 26% in milk, soft cheese, curls cheese and yogurt, respectively. Mannitol egg-yolk agar (MYP) supplemented with polymyxin B sulfate is used for isolation. Identification of the isolates was done by biochemical tests. Screening of isolates by polymerase chain reaction (PCR) revealed the presence of diarrheal toxin *cyt K* gene in 87.09 % of isolates. Susceptibility of isolates to 7 antimicrobial agents revealed neomycin, chloramphenicol and gentamycin 100% for each, streptomycin 96% and erythromycin 93.5 % to be the most effective antimicrobial, while the highest resistance was noted against penicillin 100 %.

INTRODUCTION

Bacillus cereus is an ubiquitous Gram-positive, spore-forming, motile rod, commonly found in soil, plant material, hay, raw and processed food. It is also frequently found in pasteurized milk, causing spoilage because of the production of lipase and protease (1).

It is common in dried foodstuff, spices, cereals, meat and meat products, cooked and inappropriately kept food etc. (2,3,4). It is widespread in the environment (soil, water and dust) from where it easily spreads to the foods of plant origin and through cross-contamination to other foods such as milk, meat and meat products (5,6).

B. cereus causes two distinct poisoning syndromes, i.e. diarrhea and emesis, and be responsible for some non-gastrointestinal infection (3,7).

The diarrheal type of food-born illness is caused by three different labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestinal after ingestion (8). The emetic type is due to the production of a heat-stable emetic toxin (cereulide) during growth in foods under various condition (8).

Granum (5) has reported that there are several different toxins produced by *B. cereus* : enterotoxins, haemolysin (cerolycin, haemolysin II and sphingomyelinase), phospholipase C (phosphophatidylinositol hydrolase, phosphatidylcholine hydrolase and sphingomyelinase) and emetic toxin (5).

Among the enterotoxins produced by *B. cereus* is (Cyt K) (9,10). Cyt K is a hemolytic toxin with homology to the β-barrel pore-forming toxins (11,12).

The problem in milk and milk products are caused by *B. cereus* which is spread from soil and grass to udder of cows and into the raw milk. Through sporulation *B. cereus* spores survive pasteurization, and after germination the cells are free competition from other vegetative cells (13).

Strains of food-borne bacterial pathogens that are resistant to variety of antimicrobial have become a major health concern (14,15).

Due to the hazardous of *B. cereus*, the study was designated for determine the prevalence of *B. cereus* from milk and milk products (soft cheese, curls cheese and yogurt) from Basrah local markets and detection the enterotoxin (*cyt K* gene) in these isolates by (PCR), and determine their antimicrobial susceptibility pattern.

MATERIALS AND METHODS

Samples collection

A total of 227 samples of milk, soft cheese, curls cheese and yogurt were collected from different local markets in Basrah city from September to February, 2012.

Samples were collected in sterile container, kept in ice box and transferred immediately to the laboratory. They were prepared and examined for the presence of *B. cereus* .

Isolation of *B. cereus*

The samples were transferred in peptone water (peptone water used to avoid the strain variation and keep the strain a live as possible, peptone water (oxoid) 0.1%, PH 7.0, was prepared and used as a diluent).

One ml of milk transferred to 9 ml of peptone water, and 1 gm of milk products (cheese and yogurt) transferred to 10 ml of peptone water. Then 0.1 ml was streaked on the surface of agar (MYP agar already have been prepared) spread by sterile L-shape (16). The MYP agar culture plates were incubated in the incubator at 35°C for 2 days. Gram stain and spore stain as well as colony morphology were conducted to suspected bacteria.

According to the FDA method, typical *B. cereus* colonies on Mannitol Egg Yolk Agar (MYP) supplemented with polymyxin B sulfate (Himedia), are surrounded by a precipitated zone which indicates lecithenase activity and a pink color is observed because mannitol is not fermented (17).

The typical *B. cereus* colonies (dry, rough surface; red-purple with a white precipitat) were transferred to nutrient agar slants, and confirmed by identification tests including: Gram stain, spore stain, motility, V-P test (18,19).

Bacteria were grown on nutrient agar for routine use and maintained in nutrient broth with 15% glycerol at -20°C (20).

Identification of *B. cereus*

The bacterial isolates were tested for: Motility, catalase, Voges-Proskauer reaction, starch hydrolysis, citrate utilization and hemolysin (21). Urase test and indol test also used to confirm the identify of *B. cereus* (22).

DNA extraction

Isolation of DNA

Genomic DNA for PCR was purified from bacterial cells cultured in brain heart infusion broth (oxoid) using commercial kit, and following the protocol provided by the manufacture (Geneaid). DNA samples were stored at -20°C until used.

Detection of enterotoxin gene using PCR

The oligonucleotide primer used for the detection of *cyt K* gene with the anticipated size of the amplified product is :

Cyt K F: CGA CGT CAC AAG TTG TAA CA
Cyt K R: CGT GTG TAA ATA CCC CAG TT

In all assays, PCR mixture contained AccuPower of PCR master mix (BioNeer), 1 µmol of each primer (BioNeer) and 5 µl of template DNA in a total volume of 20 µl .
Detection of the *cyt K* gene was performed as reported by (23).
The cycling conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation step at 94°C for 45 sec, annealing step at 54°C for 1 min, extension step at 72°C for 2 min, and final extension step at 72°C for 5 min.
PCR products were detected in 1.5 % agarose gel stained with ethidium bromide (0.5µg/ml), viewed by U.V. transillumination and photographed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the well diffusion method (24). *B. cereus* isolates were tested for susceptibility to 7 antimicrobial disk (Bioanalyse).
The following antimicrobs were used :-
Penicillin G (10 Units), neomycin N (30 mg), streptomycin S (10 mg), erythromycin E (15 mg), chloramphenicol (30 mg), gentamycin CN (10 mg) and tetracycline TE (30 mg).

Statistical analysis

In order to determine the statistical significances among different variables, Minitab (Statistical package for social sciences) version 15 was used.

RESULTS

A total of 227 samples were collected from local markets in Basrah city. All tested samples of each food were contaminated with *Bacillus cereus* in a ratio of 32.7% for milk, 16.6% for soft cheese, 18% for curls cheese and 26% for yogurt (Table 1). There were no significant differences ($P > 0.05$) in the rate of *B. cereus* isolation according to different samples.

The colony morphology on MYP medium was violet – red background and surrounded by an egg-yolk precipitate. The colony was flat, dry, about 5 mm in diameter.
The morphological characters were checked by Gram's and spore stain.

Table 1 Number and percentage of positive samples (milk and milk products) having *B. cereus* isolates

Sample	No. of samples	Positive sample	
		No.	%
Milk	61	20	32.7
Soft cheese	66	11	16.6
Curls cheese	50	9	18
Yogurt	50	13	26
Total	227	53	23.3
P > 0.05			

***Bacillus cereus* isolates**

The isolates shows negative results for indol and oxidase, and positive for other tests (Table 2).

Table 2 Identification of *Bacillus cereus*

Test	**Indol Test**	**Gram Stain**	**Spore stain**	**Catalas**	**Vogus-Proskaue**	**Citrate utilizatio**	**Blood hemolys**	**oxidas**	**Starch hydrolysi**	**Urase test**	**Lecithena reaction**
Result	-	+	+	+	+	+/-	+	-	+	+	+

(+) positive , (-) negative , (+/-) variable

The *cyt K* gene was detected in 27 out of 31 isolates (87.09 %), distributed in milk, yogurt, soft cheese and curls cheese in 81.81%, 90%, 80% and 100%, respectively. There were no significant differences (P > 0.05) in the presence of the enterotoxin (*cyt K*) gene in the studied samples (Table 3, Fig. 1).

Table 3 Detection of enterotoxin (*cyt K*) gene in *Bacillus cereus* isolates

Sample	Isolate	Positive Cyt K	%
Milk	11	9	81.81
Yogurt	10	9	90.00
Soft cheese	5	4	80.00
Curls cheese	5	5	100.00
Total	31	27	87.09
P > 0.05			

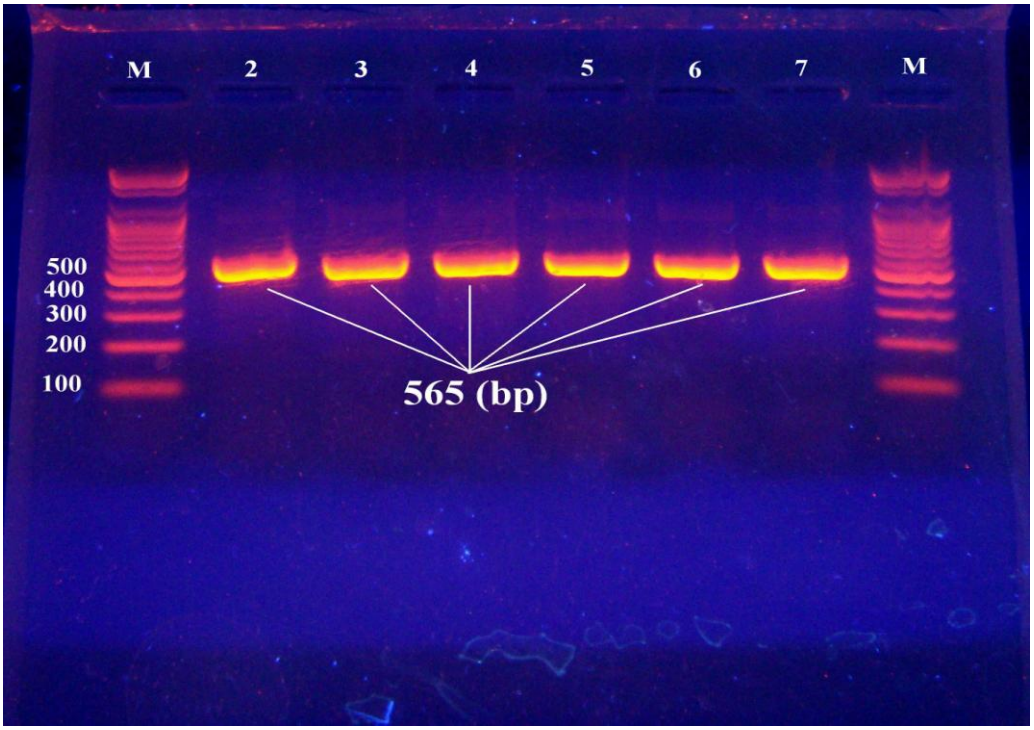

Fig. 1: PCR product of *cyt K* gene of *Bacillus cereus* isolates.
Lane 1& 8 = Molecular size marker
Lane 2-7 = *cyt K* gene

The results show that all isolates of *B. cereus* were resistance to penicillin (100%). While showed high susceptible ranging from 93.5-100%, There were significant differences (P < 0.05) between the effect of different antimicrobials against the *B. cereus* isolates (Table 4, fig. 2).

Table 4 Antimicrobial susceptibility of isolated *Bacillus cereus* strains

Antimicrobial		conc. (mg/disc)	N=31	
			S%	R%
Penicillin	P	10 U	0	100
Neomycin	N	30 mg	100	0.0
Streptomycin	S	10 mg	96	3.2
Erythromycin	E	15 mg	93.5	6.4
Chloramphenicol	C	30 mg	100	0.0
Gentamycin	CN	10 mg	100	0.0
Tetracyclin	TE	30 mg	54.8	45.1
$P < 0.05$				

S: susceptible
R: resistance

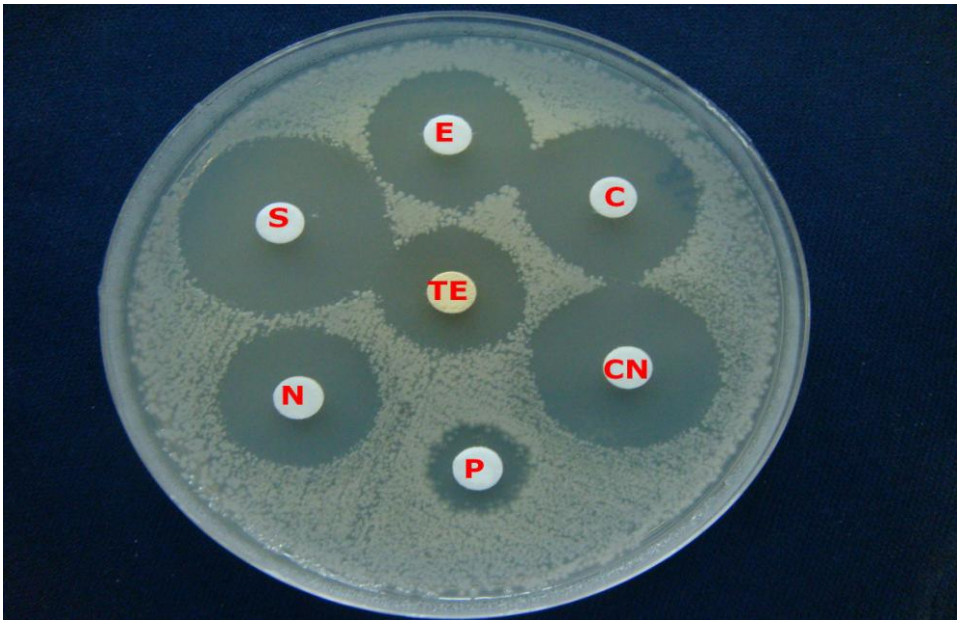

Fig. 2: Susceptibility of *B. cereus* isolates to antimicrobial agents:
P: Penicillin, N: Neomycin, S: Streptomycin, E: Erythromycin,
C: Chloramphenicol, CN: Gentamycin, TE: Tetracyclin.

DISCUSSION

Bacillus cereus is becoming an important food-poisoning organism because of its cosmopolitant distribution in nature. It was found that *B. cereus* was the most common pathogen in the raw milk (25).

This study revealed a high incidence of *B. cereus* in milk and milk products. The organism were isolated in 32.7 %, 16.6 %, 18 % and 26 % of milk, soft cheese, curls cheese and yogurt, respectively.
This result agreed with (26) and (27) who reported the high load of *B. cereus* in Nigerian foods and food condiments.
The high level of contamination by *B. cereus* may be attributed to the ubiquitous distribution of this organism in the environment (28). Storage time and storage temperatures were considered to be independent, which is obviously not true. It may be assumed that milk stored at high temperature will be stored for a shorter period simply due to spoilage (29).

In this study the enterotoxin (*cyt K*) gene was found in 27 of 31 isolates at the percentage 87.09 %. Whereas other workers detected the gene in 88, 70.40 and 29 % of their isolates, respectively (23,30,31). This result reflect the biohazardous of these isolates to the public health. Thus the milk should be treated by heating before consuming.

Our study indicates that *B. cereus* isolated were highly susceptible to chloramphenicol, neomycin and gentamycin 100% for each one.
The finding of the present study regarding susceptibilities to these agents are similar to those obtained in other countries (32,33,33,7).
In this study, the *B. cereus* isolates were highly susceptible to chloramphenicol, neomycin and gentamycin; the isolates also were susceptible to streptomycin and erythromycin. Isolates especially resistant to penicillin, streptomycin, erythromycin and tetracycline were identified. The results show susceptibility of isolates to neomycin, chloramphenicol and gentamycin 100 % for each one, and there were significant differences ($P < 0.05$) between the effect of different antimicrobials against the *B. cereus* isolates.
Previous work has shown that the antimicrobial susceptibility of *B. cereus* isolates were highly susceptible to chloramphenicol, erythromycin, streptomycin and tetracyclin and less susceptible to cotrimazole and ampicilin (35).
The variation in the value of percentage susceptibilities in this study may be attributed to the difference in the concentration of antimicrobial agents used, differences in the sources of isolates, drug resistance transfer and the wide spread use of the antimicrobials. The high percentage of isolates resistant to penicillin may be due to the frequent use of the agents in sublethal doses in medical and veterinary practices to prevent or treat infection.

The antimicrobial resistance pattern of *B. cereus* from foods is useful in epidemiological studies (36).

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الكشف الجزيئي لجين الذيفان المعوي (*cyt K*) و تحديد الحساسية الضد مايكروبية للبكتريا العصوية *Bacillus cereus* المعزولة من الحليب و مشتقاته

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الخلاصة

تم جمع مئتا وسبع وعشرون عينه من الحليب الخام والجبن الطري وجبن الظفائر والروب لعزل بكتريا *Bacillus cereus* حيث وجدت هذه البكتريا بنسبة 23.7 % في الحليب الخام و بنسبة 16.6 % في الجبن الطري و بنسبة 18 % في جبن الظفائر و بنسبة 26 % في الروب .
استعمل الوسط الزرعي الخاص مانيتول متعدد المكسين B المحتوي على صفار البيض لعزل البكتريا . تم تشخيص البكتريا باستخدام الاختبارات البايوكيميائية . باستعمال سلسلة تفاعلات البوليمريز تم تحديد جين الذيفان المعوي لعزلات *B. cereus* (*cyt K*) في 87.09 % من العدد الكلي للعزلات و تم تحديد الحساسية المايكروبية تجاه 7 اقراص من المضادات المايكروبية حيث وجد ان البكتريا اكثر حساسة تجاه المضاد الحيوي neomycin ، المضاد الحيوي chloramphenicol ، والمضاد الحيوي gentamycin بنسبة 100 % لكل منهم، والمضاد الحيوي streptomycin 96 %، والمضاد الحيوي erythromycin 93.5 % . و وجد ان البكتريا مقاومة بنسبة 100 % للمضاد الحيوي pencillin.

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