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MOLECULAR DETECTION OF TETRACYCLINE RESISTANCE GENES IN *Bacillus cereus* ISOLATED FROM FOOD SOURCES

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ABSTRACT

Two hundreds of different food samples i.e. cream, beef meat, frozen beef meat, burger, cooked rice and minced rice were collected from local markets in Basrah city. The tested samples of each food type were found to be contaminated with *Bacillus cereus* (*B. cereus*) in a ratio of 36.36 % for cream, 26.47 % for beef meat, 50 % for frozen beef meat, 36.36 % for burger, 18.18 % for cooked rice and 51.51 % for minced rice. Mannitol egg-yolk agar (MYP) supplemented with polymyxin B sulfate is a selective media used for isolation. Identification of the isolate was done by detection of 16SrDNA and conformed by sequencing. Antimicrobial susceptibility test was used for screening the isolates, which were resistance to tetracycline. *Bacillus cereus* isolates were resistance to tetracycline TE 30 mg/disc (100 %) in cream and (0 %) in cooked rice. Polymerase chain reaction was used for detection of the presence of tetracycline resistance gene in the percentage 32 % and 44% for *tet (*K) and *tet* (L), respectively.

INTRODUCTION

Bacillus cereus is a gram positive, rod shaped and spores forming bacterium that causes severe food poisoning in human. The spores can stay alive in hot and dry conditions, and remain dormant for many years (1). *B. cereus* causes two types of food poisoning in humans: the diarrhoeal (thermo labile toxin) and emetic (thermo stabile toxin) type. Both types can seriously affect human health (2), causing severe infections including sepsis, meningitis, endocarditis, endophthalmitis, respiratory and surgical wound infections (3). Recently *B. cereus* was connected to hospital infection (4). Diarrheal disease has been a major public health problem in several countries causing high morbidity and mortality among children (5). Tetracycline (TC) is a broad-spectrum antibiotic used in the treatment of bacterial infections in humans, animals, and insects. Therefore, bacteria from different ecosystems are exposed to this antibiotic leading to antibiotic resistance (6). Three main mechanisms of resistance to tetracycline that is, energy-dependent efflux, protection of the bacterial ribosome, and enzymatic inactivation of the

tetracycline molecule (6). *B. cereus* have been shown to carry the *tet(L)* gene on a plasmid, while other species of *Bacillus* carry either *tet(L)* or *tet(K)* on plasmids, and/or in the chromosome (7-9). Both of these genes can on occasion be mobilized in the presence of conjugative plasmids but are not themselves able to independently transfer thus slowing their spread within the population. The *tet(K)* and *tet(L)* genes encode efflux proteins which pump tetracycline and doxycycline out of the cell. These genes are commonly found in specific Gram-positive genera (10). The aim of this study is the isolation and identification of *Bacillus cereus* isolates from different food samples which cause food poisoning and determinates the tetracycline resistance genes in these isolates.

MATERIALS AND METHODS

Samples collection

A total of 200 samples of cream, beef meat, frozen beef meat, rice cereals, cooked rice and burger were collected from different local markets in Basrah city from February to April, 2018. All samples were collected in sterile container, kept in icebox and transferred immediately to the laboratory where they were prepared and examined for the presence of *Bacillus cereus* (11).

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 gram from each sample i.e. burger, cream, beef meat, frozen beef meat, rice cereals and cooked rice transferred to 10 ml of 0.1 % peptone water. Then 0.1 ml was streaked on the surface of MYP agar, spread by sterile L – shape. The MYP agar culture plates were incubated at 35 °C for 2 days (12). Colony morphology and Gram's stain were conducted to suspected bacteria. A 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 (13).

Identification of *B. cereus*

The identification is done by 16S rDNA partial sequence (14).

DNA Extraction

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.

PCR Amplification

The amplification of the 16S rDNA is done by (PCR) using universal primers and PCR conditions described by (15). The oligonucliotide primers which were used have 1541 bp and their sequence are :

16S rDNA F: AGAATTTGATCCTGGCTTAG

16S rDNA R: AAGGAGGTGATCCAGCC

PCR mixture contained 25 µl of PCR green master mix (Promega), 2 µl of each primer (BioNeer) and10 µl of template DNA in a total volume of 50 µl with free water. The cycling conditions were initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation step at 94°C for 1 min., annealing step at 54.7°C for 1 min, extension step at 72°C for 1min, and final extension step at 72°C for 10 min (15). PCR products were detected in 1.5 % agarose gel stained with ethidium bromide (0.5µg/ml), viewed by U.V. transillumination and photographed (15).

Tetracycline resistance test

The tetracycline resistance testing was determined by the disk agar diffusion method (16). *B. cereus* isolates were tested for resistance to tetracycline disk 30µg (Bioanalyse).

Detection of *tet (K) and tet (L)* genes by PCR

Tetracycline resistance genes *tet (K)* and *tet (L)* were detected by PCR. The primers used in this study were:

***tet (K)* F : TCG ATA GGA ACA GCA GTA,**
***tet (K)* R : CAG CAG ATC CTA CTC CTT**
***tet (L)* F : TCG TTA GCG TGC TGT CAT TC**
***tet (L)* R: GTA TCC CAC CAA TGT AGC CG**

These primers are for forword and reverse for tet (K) and tet (L), respectively (17). PCR mixture contained 12.5 µl of PCR green master mix (Promeg), 1 µl of each primer (BioNeer) and 5 µl of template DNA in a total volume of 25 µl with free water. For *tet (K)* gene, the cycling conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation step at 94°C for 1 min., annealing step at 48°C for 1 min, extension step at 72°C for 1.5 min, and final extension step at 72°C for 10 min. For *tet (L) gene*, the cycling conditions were initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation step at 94°C for 1 min., annealing step at 56°C for 1 min, extension step at 72°C for 1min, and final extension step at 72°C for 10 min (17). PCR products were detected in 1.5 % agarose gel stained with ethidium bromide (0.5µg/ml), viewed by U.V. transillumination and photographed.

RESULTS

A total of 200 samples were collected from local markets in Basrah city. The tested samples of each food were contaminated with *B. cereus* in a ratio of 36.36 % for cream, 26.47 % for beef

meat, 50 % for frozen beef meat, 36.36 % for burger, 18.18 % for cooked rice, and 51.51 % for minced rice (Table 1).

Table (1) Number and percentage of Cream, Beef meat, Frozen beef meat, Burger, Cooked rice and Minced rice having *B. cereus* isolates

Sample	No. of samples	Positive sample	
		No.	%
Cream	33	12	36.36
Beef meat	34	9	26.47
Frozen beef meat	34	17	50
Burger	33	12	36.36
Cooked rice	33	6	18.18
Minced rice	33	17	51.51
Total	200	73	36.5
P < 0.05			

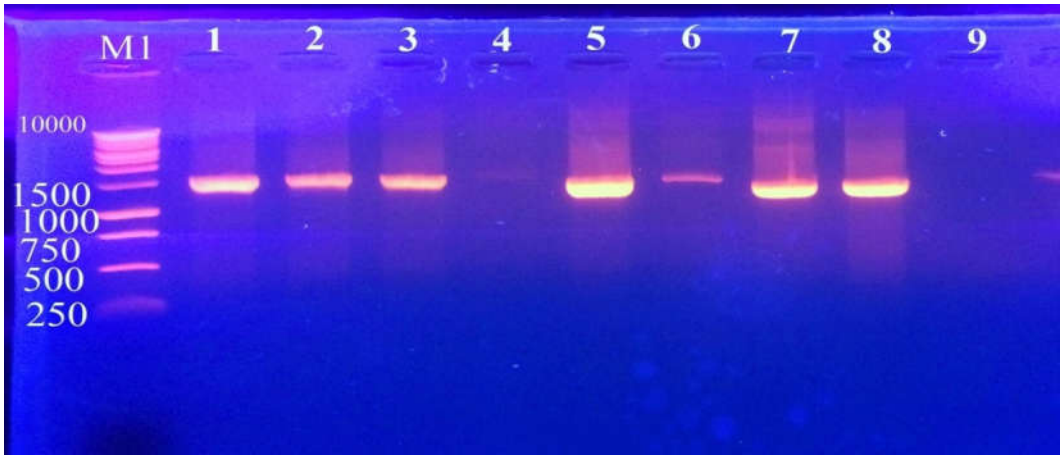

Figure1: Identification of *Bacillus cereus* by detection of 16S rDNA

Lane M1= Ladder, Lane = 1 – 8 positive for 16S rDNA 1541 bp, Lane 9 = negative control.

The colony morphology on MYP medium was violet – red background and surrounded by an egg-yolk precipitate. In microscopic examination, the bacterium is gram positive, rod bacilli and spore forming. All *B. cereus* isolates distributed in cream, beef meat, frozen beef meat, burger,

cooked rice, minced rice, were identified by 16S rDNA with similarity of 80%-100% (Table2**)** when it blast in the NCBI data base (Figure 1) shown the amplicons of *B. cereus* isolates using 16s rDNA universal primers which produced 1541 bp in size.

Table 2 Blast analysis of DNA sequencing of 16rRNA of bacterial strains

Sample No.	Species	Accession	Identity %
S1	*Bacillus cereus*	EU346663.1	97
S2	*Bacillus cereus*	CP009641.1	98
S3	*Bacillus cereus*	KY750689.1	95
S4	*Bacillus cereus*	LC189362.1	97
S5	*Bacillus cereus*	MH552993.1	96
S6	*Bacillus cereus*	KJ870036.1	92
S7	*Bacillus cereus*	KY750690.1	95
S8	*Bacillus cereus*	CP009641.1	95
S9	*Bacillus cereus*	MG711894.1	97
S10	*Bacillus cereus*	LC189361.1	95
S11	*Bacillus cereus*	MH571498.1	94
S12	*Bacillus cereus*	EU346663.1	95
S13	*Bacillus cereus*	KY750689.1	95
S14	*Bacillus cereus*	MH571498.1	94

Bacillus cereus isolates were resistance to tetracycline TE 30 mg/disc (100 %) in cream and (0%) in cooked rice. Table 3, Figure 2.

Table (3) Antimicrobial susceptibility of *Bacillus cereus* isolates against tetracycline antimicrobial disk

The isolates type	No. of isolates	N= 39 No. of isolates			
		No. of S	S%	No. of R	R%
Cream	6	0	0%	6	100 %
Beef meat	6	4	66.66 %	2	33.33 %
Frozen beef meat	5	3	60 %	2	40 %
Burger	7	1	14.28 %	6	85.71 %
Cooked rice	5	5	100 %	0	0 %
Minced rice	10	1	10 %	9	90 %
Total	39	14	35.89 %	25	64.10 %
P < 0.05					

S: susceptible , R: resistance

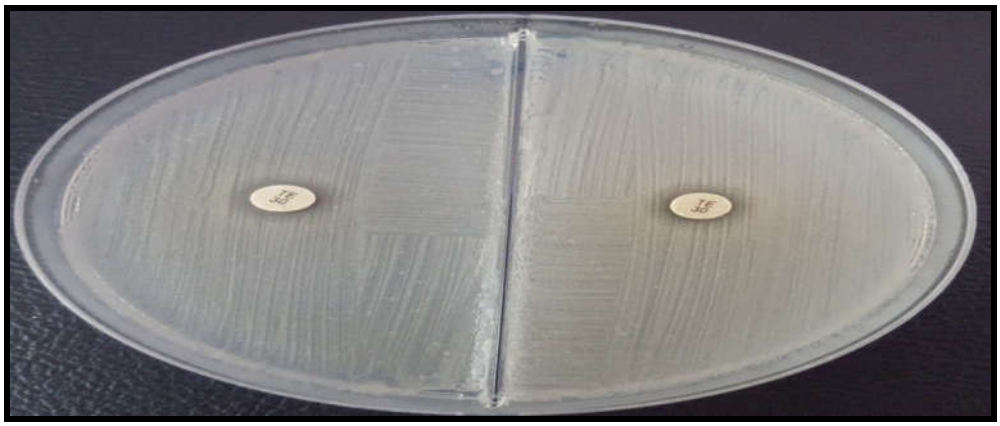

Figure2: Tetracycline resistance test

Tetracycline resistance gene investigations were done by PCR. *Tet (K) gene* was detected in 8 out of 25 isolates (32 %), distributed in cream, beef meat, frozen beef meat, burger and minced rice in 50%, 0%, 100%, 33.33% and 11.11%, respectively. But this gene was not detected in both of frozen beef meat and cooked rice. Table 4, Figure 3

Table (4) Detection of tetracycline resistance (*tet K*) gene in *Bacillus cereus* isolates

Sample	Isolate	Positive tet K	%
Cream	6	3	50 %
Beef meat	2	2	100 %
Frozen beef meat	2	0	0 %
Burger	6	2	33.33 %
Minced rice	9	1	11. 11 %
Total	25	8	32 %
P > 0.05			

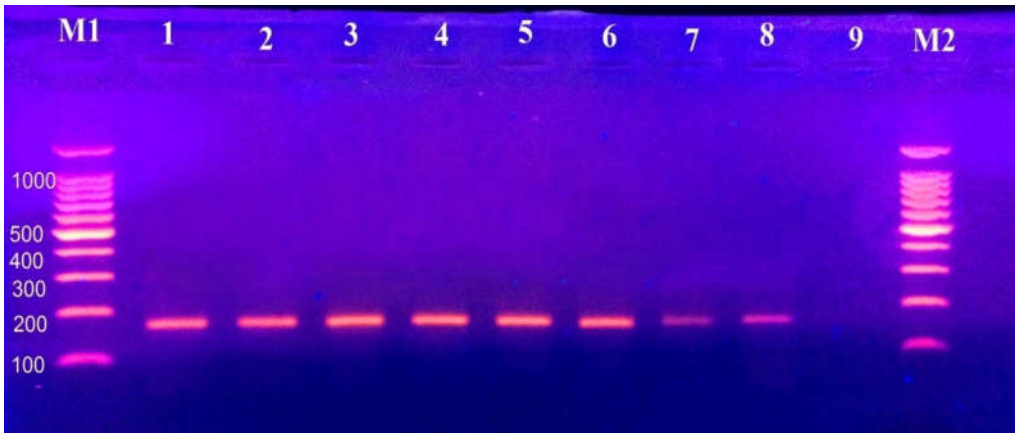

Figure3: Detection of tetracycline resistance gene *tet (K).*

Lane M1, M2 = ladder, Lane 1-8 = positive for *tet K* gene 169 bp, Lane 9= negative control

Tet (L) gene was detected in 6 out of 25 isolates (44 %), distributed in cream, beef meat, frozen beef meat, burger and minced rice in 33.33%, 50% 50%, 83.33% and 22.22%, respectively. But this gene was not detected in cooked rice. Table 5, Figure 4.

Table (5) Detection of tetracycline resistance *tet(L)* gene in *Bacillus cereus* isolates

Sample	Isolate	Positive tet L	%
Cream	6	2	33.33 %
Beef meat	2	1	50 %
Frozen beef meat	2	1	50 %
Burger	6	5	83.33 %
Minced rice	9	2	22.22 %
Total	25	11	44 %
P > 0.05			

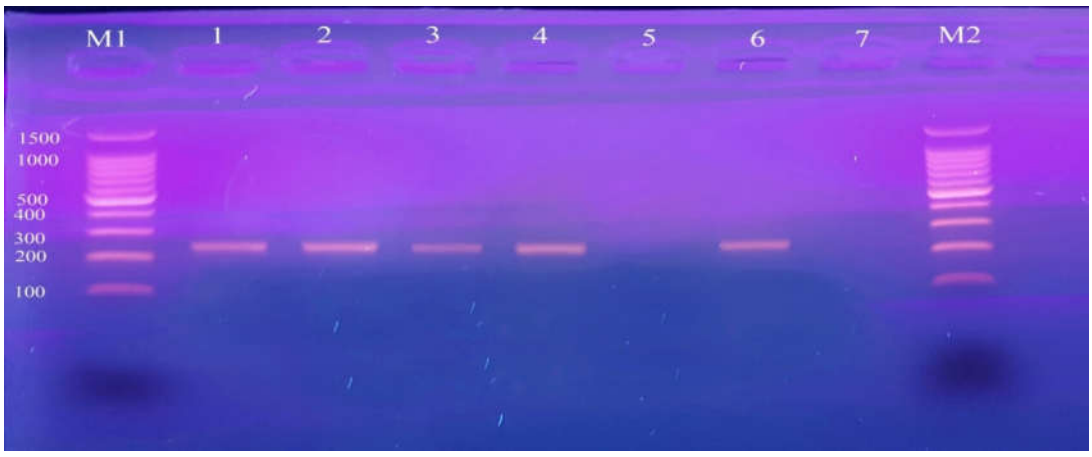

Figure 4: Detection of Tetracycline resistance gene *tet (L)*

Lane M1, M2= Ladder, Lane 1- 4, 6= positive for *tet (L)* gene 267 bp, Lane 5= negative for *tet (L)* gene, Lane 7= negative control

DISCUSSION

Bacillus cereus is an opportunistic pathogen causing severe food poisoning. The poisoning manifested by diarrhoeal or emetic syndrome (18). This study revealed a high incidence of *B. cereus* in food sources in a percentage of 36.36 %, 26.47 % , 50 %, 36.36 % , 18.18 % , and 51.51 % for cream, beef meat, frozen beef meat, burger , cooked rice and minced rice , respectively. The result agreed with Schlegelova *et. al,.* 2003, who reported that the contamination with *B. cereus* strains was recorded in 31% of all dairy products (66 samples), and in 28% of samples (31 samples) of meat products (18). The recorded prevalence of strains in the foodstuffs examined is not at variance with characteristic properties of *B. cereus*. That is also reported that products made from skimmed milk (curd cheese) were contaminated in one case only (3.2% of products), while products with higher fat content (even if made with different production technologies) in 10–16% of cases (19).

Antibiotic resistance is a major health problem during past time. It developed from resistance to single classes of antibiotics to multidrug resistance and extreme drug resistance. Spreading of resistance genes due to their relocation from the chromosomes of environmental bacteria to a mobile element and then to clinical pathogens (20- 24). The antibiotics are getting worth now days and due to number of clinical concerns their usage is important and there is still need of research for the new discoveries (25).

Our study indicates that *Bacillus cereus* isolates were resistance to tetracycline TE 30 mg/disc (100 %) in cream and (0 %) in cooked rice. A relatively higher phenotypic resistance against tetracycline (64.10%) was noted compared to the studies of Schlegelova *et. al.,* (26,27) and Whong and Kwaga (28), who found 3.03% and 6.7% of *B. cereus* isolates resistance against tetracycline, respectively, therefore the isolates were screened for presence of tetracycline resistance genes. Ankolekar *et. al.,* (29), reported tetracycline resistance in 49 of the strains (98%), and also Chaves *et. al.,* (30) also found predominant resistance to tetracycline, whereas a total of 26 strains were found to be susceptible to tetracycline with a rate of 89.7% (31).

This project could be easily extended to other classes of antibiotic resistance genes to understand the pathways leading to acquisition of drug resistance by human- and animal-pathogenic bacteria (32). The main goal of this study is to continuing the previous studies (33,34,35) and isolation and identification of *Bacillus cereus* from different samples and determinates the tetracycline resistance genes in these isolates.

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