Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 8-20

Detection of Enterotoxin (*hblA,hblC* and *hblD*) Genes of *Bacillus cereus* Isolates from Different Food Samples Using Traditional and Molecular Methods

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Received: March 11, 2015 / **Accepted**: March 29, 2015

Abstract: A total of 245 samples of foods (pasteurized milk, cheese, yoghurt, meat, and coffee) collected from different local markets at Baghdad city. The samples were cultured on selective media , polymyxin B pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) to isolate *B.cereus*. Results revealed that the isolation of fifty five *B. cereus* isolates distributed as 26.2 % in pasteurized milk , 22.8% in cheese, 30.2% in yoghurt, 15.6% in meat and 8% in coffee. To confirm the identification, all the fifty five *B.cereus* isolates were undergone to biochemical test , and grown on blood agar . PCR amplification resulted in the occurrence of each of the genes *hblA, hblC and hblD* in the percentages of 38.2%, 60% and 60% respectively out of the total number of isolates.

Key words: *Bacillus cereus* , polymyxin B , pyruvate egg yolk mannitol bromothymol blue agar, *hblA* ,*hblC* and *hblD* , Food Samples

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Introduction

Foodborne illness (also foodborne disease and colloquially referred to as food poisoning) is any illness resulting from the consumption of contaminated food with pathogenic bacteria, viruses and parasites or their toxins as well as chemical or natural toxins such as poisonous mushrooms (1). The genus *Bacillus* is a large and diverse group of bacteria belonging to the family Bacillaceae, Phylum Firmicutes. The species in this genus are aerobic or facultatively anaerobic, endospore forming, rod shaped gram positive bacteria that are widely distributed in nature, especially in the soil. *Bacillus cereus* known as the causative agent of food-borne illness belongs to Group I of the genus *Bacillus*. Other species in this genus including *B. anthracis*, *B. subtilis*, *B. licheniformis*, *B. thuringiensis* and *B. pumilus* are increasingly recognized as food poisoning agents (2). *B. cereus* is widely distributed and approximately found in every environment. This means that it is easily transmitted into the food chain. The spores formed by *B. cereus* are highly resistant against environmental stresses, and are thus able to survive through several processing conditions used in food industry (3). The unique properties of *B. cereus* are endospores which have a more hydrophobic surface than any other *Bacillus spp*. spores. They adhere

to surfaces such as steel and plastics, they are difficult to remove during cleaning. The survival and growth at different temperatures (low to high) and toxigenic potential have directed the focus of research towards understanding phenotypic and genotypic characteristics of this bacterial species known to cause health hazards. The changing scenario of food habits has given impetus on the use of processed foods with minimal processing and prolonged storage at low temperatures (4). *B. cereus* is the etiological agent of two distinct types of food poisoning. One is the diarrheal syndrome, which is characterized by abdominal pain, profuse watery diarrhea and rectal tenesmus. The diarrheal syndrome caused by *B. cereus* is mediated by one or three diarrheal enterotoxins: the tripartite toxins haemolysin BL (HBL) and non-haemolytic enterotoxin (Nhe), the two forms of cytotoxin K (cytK-1 and cytK-2) and possibly enterotoxin T and enterotoxin FM (5). HBL is a three-component toxin consisting of two lytic proteins, L1 and L2, that are encoded by *hblD* and *hblC* genes respectively, and a binding component B encoded by *hblA* gene. The presence of all three components is necessary for the toxin activity (6). The distribution of *hbl* genes within the *B. cereus* species is quite diverse, and strains show different capability of producing diarrheal toxins. Several factors can group such strains based on growth temperature, food matrix and nutritional availability (7).

Materials and Methods

Samples Collection

A total of 245 samples of (pasteurized milk, cheese, yoghurt, meat, and coffee) were collected from different local markets in Baghdad city from December 2013 to June 2014. 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 pasteurized milk and yoghurt transferred to 9ml of peptone water, and 25 gm of meat , coffee or cheese and reconstituted with 225 ml of peptone saline solution (PSS) and homogenized by blender for 3 min. under sterile condition. A volume of 10 ml of the homogenate placed in a circulating water bath at 90 °C for 10 min., this heat treatment was made to destroy vegetative bacteria and fungi and to make easier the isolation of *B. cereus* from its spores that are thermo resistant, then cooled in ice bath. Then 0.1 ml was streaked on the surface of blood agar and *B. cereus* agar (PEMBA agar already have been prepared) spread by sterile L-shape , and incubated at 35°C for 24- 48 hours (8). The typical *B. cereus* colonies (Blue, Dull, Crenate, Flat, Irregular, Undulated Margin, Large size 3 – 4 mm and surrounded by a zone of precipitation, clear zone on the border of precipitation) (9). Bacteria were grown on nutrient agar for routine use and maintained in nutrient broth with 15% glycerol at -20°C (10).

Identification of *B. cereus*

The bacterial isolates were tested individually by morphological, cultural and biochemical characteristics according to the documented procedures for: Gram stain, Spore Stain, Motility, catalase, Voges-Proskauer reaction, starch hydrolysis, Citrate utilization and Hemolysin (11). Urase test and indol test also used to confirm the identification of *B. cereus* (12).

DNA Extraction

Genomic DNA 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 Genes Using PCR

The oligonucleotide primer used for the detection of *hblA, hblC* and *hblD*, genes with the anticipated size of the amplified product(13) is shown in Table.1.

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 *hblA, hblC, hblD*, genes was performed as reported by MacFaddin (14) (Table2) **.** PCR products were detected in 1.5 % agarose gel stained with ethidium bromide (0.5µg/ml), viewed by U.V. transillumination and photographed.

Table 1: Oligonucleotide primers sequences used for PCR amplification of diarrheal toxin genes

Target gene	Oligonucleotide sequence (5'–3')	Product size (bp)	Reference
hblA	F: GTGCAGATGTTGATGCCGAT R: ATGCCACTGCGTGGACATAT	320	Hansen and Hendriksen (13)
hblC	F: AATGGTCATCGGAACTCTAT R: CTCGCTGTTCTGCTGTTAAT	750	Hansen and Hendriksen (13)
hblD	F: AATCAAGAGCTGTCACGAAT R: CACCAATTGACCATGCTAAT	430	Hansen and Hendriksen (13)

Table 2: PCR amplification programme for detection of 3 genes (hbl , *hblA,hblC,hblD*)

Steps	Temperature (°C)	Time	No. of cycle
Initial Denaturation	94 °C	2 min.	1
Denaturation	94 °C	30 sec.	35
Annealing	48°C Zone 52°C 55 °C	30 sec.	
Extension	72°C	90 sec.	
Final Extension	72 °C	2 min.	1

Results and Discussion

Isolation and Identification

A total of 245 food samples were collected randomly from restaurants , retail food stores and various sale point in the local markets of Baghdad city and cultured on polymyxin B pyruvate egg yolk mannitol bromothymol blue agar (PEMBA), for detecting and isolating of *B. cereus.* The result of the present study was showed that 55 out of 245 samples were positive for *B. cereus* with a percentage of 22.45%. fifty five isolates were found to be a gram-positive, endospore forming, motile and rod shape (either a single rod or in chains). The spore was central and ellipsoidal. The colony morphology on, (PEMBA) medium was produced large (3.7 mm diameter), crenated or fimbriated to slightly rhizoid colonies. They had flat , dry distinct turquoise to peacock blue colour due to the absence of mannitol fermentation, and they were surrounded by a zone of egg-yolk precipitation caused by lecithinase activity (Figure 1). The isolates were appeared resistant to certain concentrations of Polymyxin which inhibited the other bacteria, and is effective mainly against gram-negative organisms. Colonies on blood agar were gray, large, circular, shiny, and became irregular and dry after 48 hrs. Positive colonies were subcultured on blood agar to check the hemolysis characterization (fig.2). All tested samples of each food were contaminated with *Bacillus cereus* in a ratio of 26.2 % for pasteurized milk, 22.8 % for cheese, 30.2 %for yogurt, 15.6 % for meat and 8 % for coffee (Table 3). The morphological characters were checked by Gram's and spore stains.

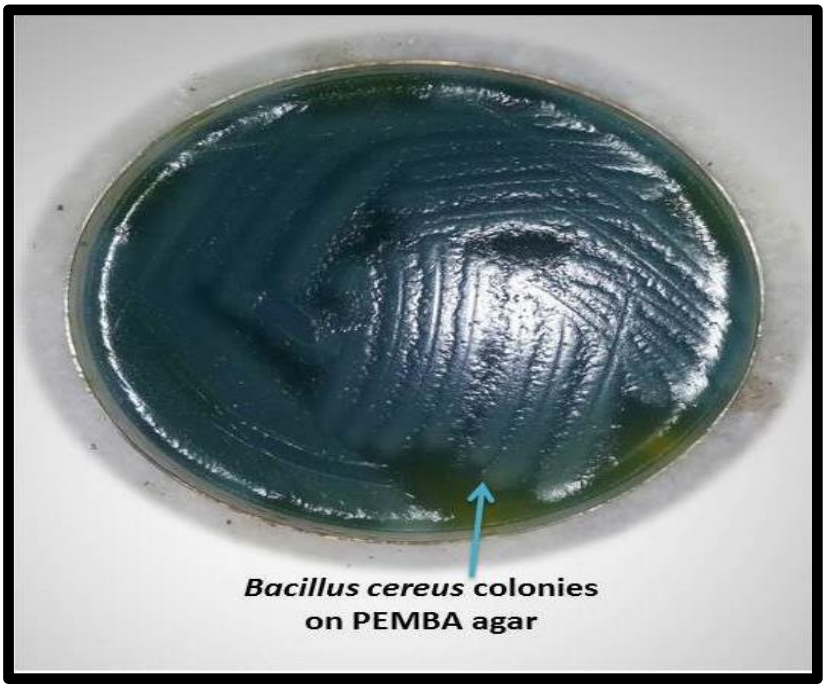

Figure 1: Native isolate of *B. cereus* streaked on to selective plate of PEMBA (the pH indicator is bromothymol blue)

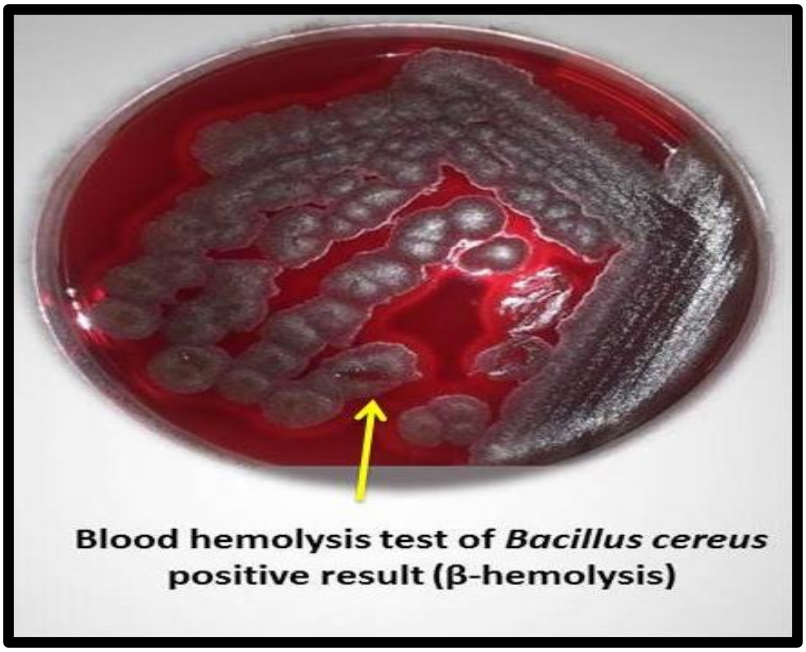

Figure 2: Blood hemolysis test of *Bacillus cereus*: positive result (β-hemolysis)

Table 3: Number and percentage of positive samples (pasteurized milk, cheese, yoghurt, meat, and coffee) having *B. cereus* isolates

Samples	No. of samples	Positive sample	
		No.	%
Pasteurized Milk	65	17	26.2
Cheese	57	13	22.8
Yoghurt	53	16	30.2
Meat	45	7	15.6
Coffee	25	2	8
Total	245	55	22.45

To confirm the identification of the 55 suspected *B.cereus* isolates , they were undergone for biochemical test . Biochemical confirmation can be based on an isolates ability to produce acid from glucose and not from mannitol , xylose and arabinose, (Table.4).

Table 4: Results of biochemical tests used for identification of *B. cereus*

Test	Result
Catalase	+
Citrate Utilization	+
Oxidase test	+
Gelatin liquefaction	+
Glucose	+
Grow in 7% NaCl	+
Indole production	-
Mannitol	-
Methyl red	+
Nitrate reduction	+
Starch hydrolysis	+
Urease activity	-
Vogas Proskauer	+

Blue color in PEMBA plates indicated that the basic medium of mannitol was not fermented by *B. cereus* as well as precipitate egg-yolk surrounding the colonies. These results agreed with the results recorded by McKillip (15), who investigated the presence of lecithinase (phospholipase Q), and the absence of mannitol fermentation in *B. cereus* that produced blue color colonies and showed the precipitation of egg yolk. PEMBA media contain polymyxin to inhibit the growth of competitive organisms. These results were in accordance with Abdul-Hadi *et a.l* (16). The suspected isolates were stained with Gram's stain and examined under the microscope. Then after treatment with dye cells of *B. cereus* appeared violet (Positive Cram stain), rod-shaped, body- single, pairs or chain cells with circular or square ends of different lengths, but when treated with malachite green stain or spores dye , the cells were stained red, while spores stained in green, and the spores were oval with a central location without causing swelling. This was consistent with what indicated by studies on *B. cereus* (17). Positive colonies were subcultured on blood agar to check the hemolysis characterization. For further confirmation, positive cultures were kept on nutrient agar slopes .The hemolysis indicates that the strains isolated from different food samples in this study could be a virulent strain (18). *B. cereus* currently attracted increasing attention due to their capability of producing a range of enterotoxins and tissue degrading enzymes (18). The present study revealed a high occurrence of *B . cereus* in (cheese , pasteurized milk , Yogurt , meat, Coffee) .Yousuf *et al., (*19) , reported the high occurrence of *B. cereus* in Nigerian foods and food condiments. Also Batchoun *et al*. (20) showed a relatively high occurrence (23.3 %) of *B.cereus* in the food samples examined. A similar level of prevalence (40-45%) as observed in this study was reported by Reyes *et al*. (21), who found the percentage of prevalence in retail chicken products, dried milk products and raw processed vegetables, respectively. The isolation of *B. cereus* from a diverse range of foods indicates the versatility of this pathogen. Similarly, strains of *B. cereus* have been isolated from a variety of sources like pulses, rice and rice products, oils, fish, meat, spices and milk and milk products (22). In this study, *B. cereus* was detected in 22 .45% of samples. (cheese , pasteurized milk , Yogurt , meat, Coffee) which is in agreement with that of Safee (23).

DNA Extraction from *Bacillus cereus*

Bacillus cereus is a gram positive bacterium. Its thik peptidoglycan layer makes DNA extraction an expensive and difficult task .DNA was extracted from *B. cereus* isolates by using Genomic DNA Mini kit supplied by the manufacturing company (Geneaid, Korea) (24) (Figure 3).

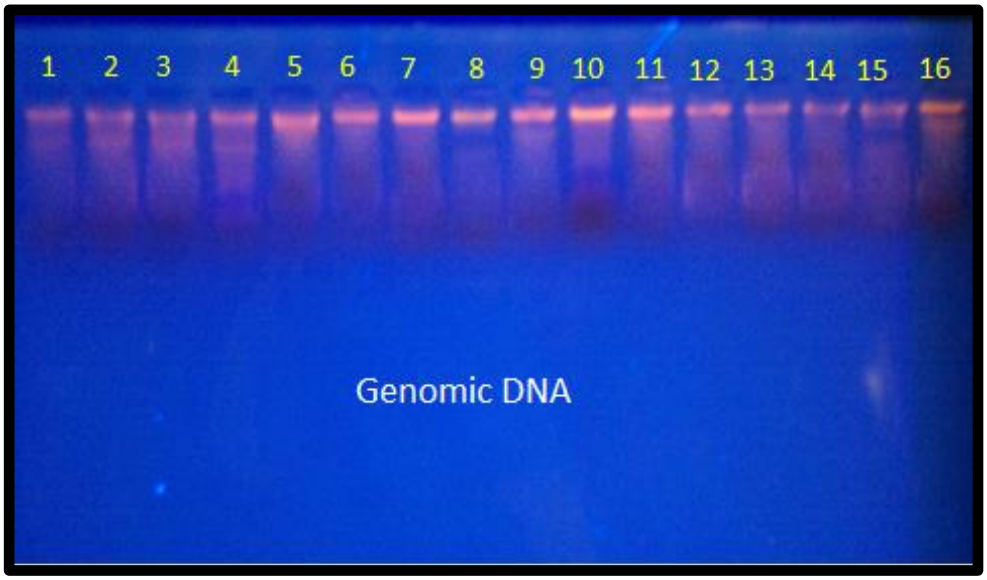

Figure 3: DNA bands extracted from *B.cereus* isolates using Genomic DNA extraction Kit (agarose 1%, TBE buffer (1X), 5V/Cm for 1 hr stained with ethidium bromide)

Detection of *hbl* Genes by PCR

PCR has become one of the most important molecular diagnostic methods for detection of food borne pathogens and is considered to be a valuable alternative to the culture-based detection techniques due to its speed, limit of detection(LOD) ,sensitivity and specificity (25). The result showed that 30. 9% (17/55) of *B. cereus* isolates contained three enterotoxic HBL complex encoding genes *hblA*, *hblC* and *hblD* , while 29% (16/55) of isolates contained two *hbl* genes, while 7.3% (4/55) of isolates with only one HBL gene, and the rest 32.7% (18/55) had no HBL genes **(** Table 5).

Table 5: Detection of *hblA, hblC* and *hblD* genes in *Bacillus cereus* isolates from food samples

Genes	Milk n=17	Cheese n=13	Yogurt n=16	Meat n=7	Coffee n=2
hblA	7(41.2%)	5(38.5%)	5(31.3%)	3(42.9%)	1(50%)
hblC	10(58.8%)	8(61.5%)	9(56.3%)	4(57.14%)	2(100%)
hblD	10(58.8%)	8(61.5%)	9(56.3%)	4(57.14%)	2(100%)
hblA,C,D	6(35.3%)	4(30.8%)	4(25%)	2(28.8%)	1(50%)
hblC,D	4(23.5%)	4(30.8%)	5(31.3%)	2(28.6%)	1(50%)
hblA	1(5.9%)	1(7.7%)	1(6.3%)	1(14.3%)	0

There is a similar study by Guoping *et al*. (26), and another study for the HBL encoding genes, they found 23 [35.6%] isolates carrying simultaneously the *hblACD* genes, 37 (58.7%) isolates, they are positive for at least one of them and 26 [41.3%] isolates that didn't harbor any of the tested genes (27). The presence of six enterotoxic genes was detected by PCR in all *B. cereus* isolates taken from coffee samples .Of all 17 strains, 100%

were positive for at least 1 enterotoxin gene; 52.9% (9/17) were positive for the 3 genes encoding the HBL complex (28) . This study was detected all *B. cereus* strains carried the HBL genes by 67.3% of strains . It was also reported that , the occurrence of the enterotoxins *hblA* ,*hblC* and *hblD* were detected in 38.2 %, 60 %, 60 % respectively (Figures -4,5,6,7,8). Yang *et al*. (29), reported similar results , they discovered that all *B. cereus* strains carried the *hbl* genes by 46.8% of strains . There is a similar study Individually, *hblA* gene was detected in 26 [41.3%] isolates, 34 [54%] isolates were positive for *hblC* gene and the same result was observed for *hblD* gene (27). Many studies have already been done to evaluate the occurrence of pathogenic genes of *B. cereus*, as that were developed by Rather *et al*., (30) in India who addressing raw and pasteurized milk and the detection of HBL genes. In that study the percentages of detection for *hblA, hblC* and *hblD* were around 70% of the tested samples . In multiplex PCR system technique, three primer pairs for *hblA, hblC* and *hblD* were included . All primer pairs used in this study (fig8) showed the appearance of the amplified products of *hblC* gene (750bp) and *hblD* gene(430bp) and the absence of *hblA* gene product(320 bp),this study was similar to previous study carried out by Ozgur *et al*., (31).

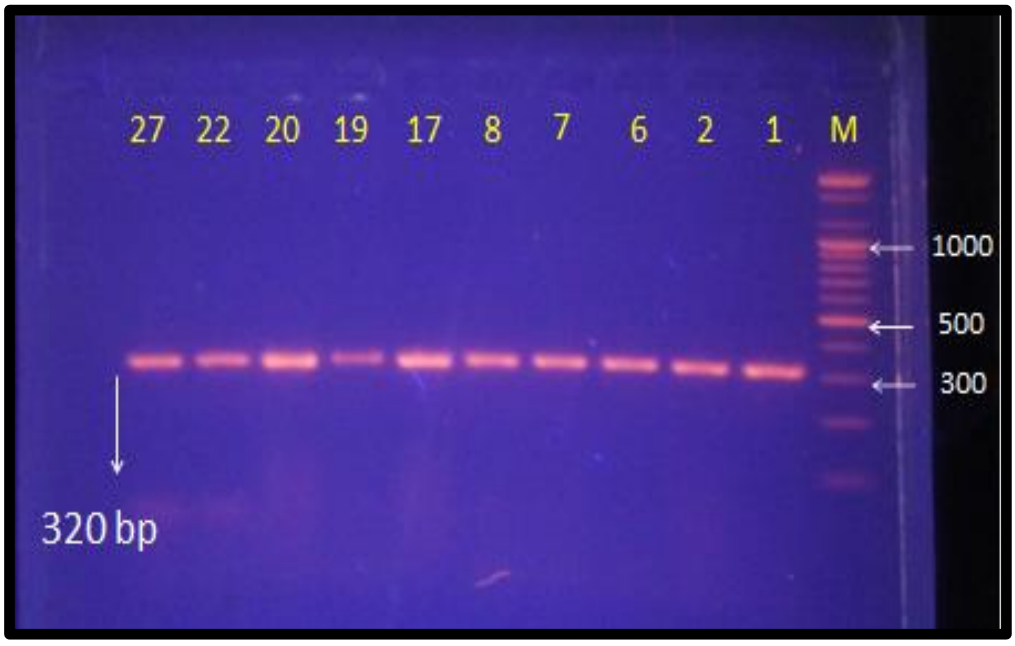

Figure 4: Agarose gel electrophoresis of PCR amplification products of *B.cereus* ,*hblA* gene (1.5% agarose, TBE buffer 1X, 5V/Cm, 2hr). M:The DNA molecular wight marker (100 bp ladder). ;lanes (1,2,6,7,8,17,19,20,22,27 represent isolates number) positive amplification of 320 bp for *hblA* gene.

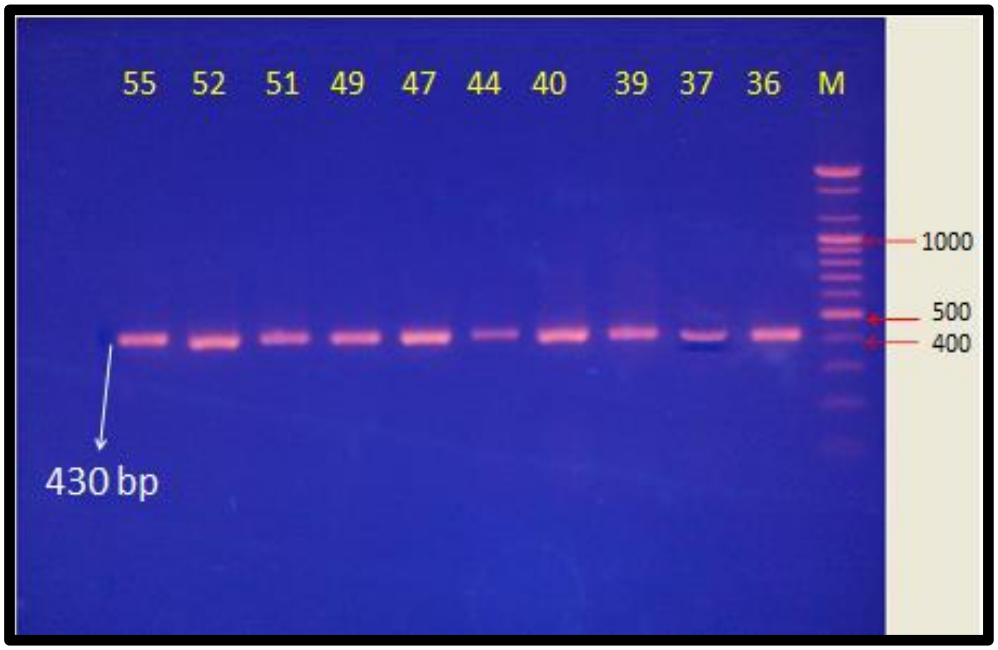

Figure 5: Agarose gel electrophoresis of PCR amplification products of *B.cereus* , *hblD* gene (1.5% agarose, TBE buffer 1X, 5V/Cm, 2hr). M:The DNA molecular wight marker (100 bp ladder). ;lanes (36,37,39,40,44,47,49,51,52,55 represent isolates number) positive amplification of 430 bp for *hblD* gene

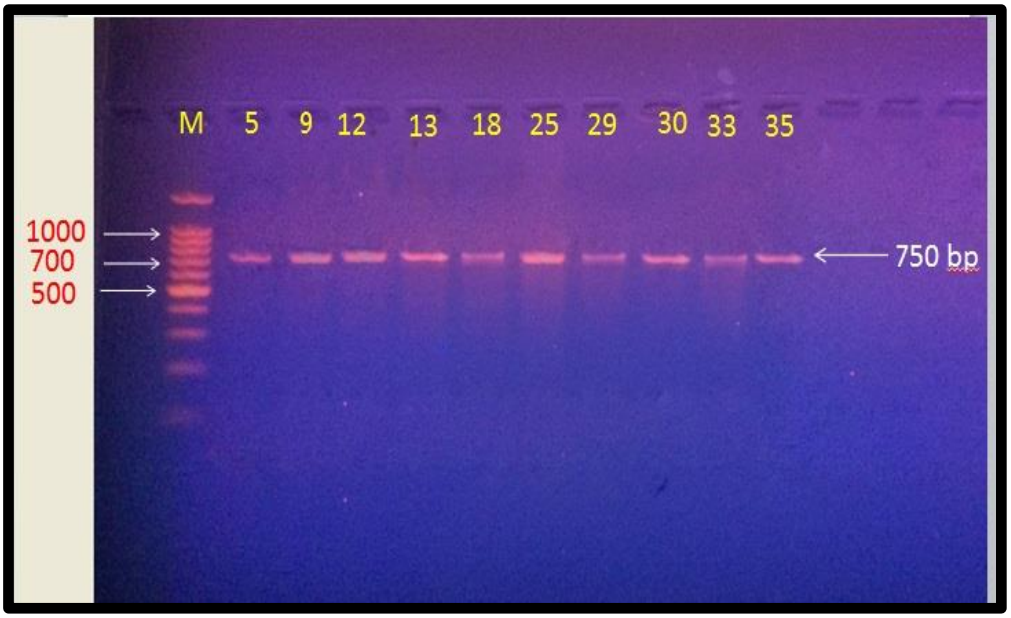

Figure 6 :Agarose gel electrophoresis of PCR amplification products of *B.cereus* , *hblC* gene (1.5% agarose, TBE buffer 1X, 5V/Cm, 2hr). M:The DNA molecular wight marker 100 bp ladder. ; lanes (5,9,12,13,18,25,29,30,33,35 represent isolates number) positive amplification of 750 bp for *hblC* gene

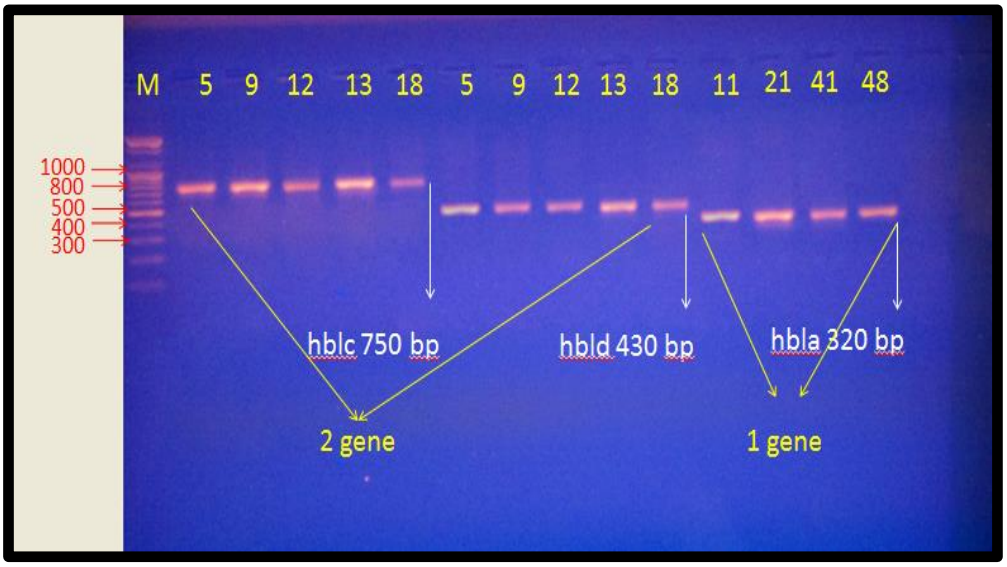

Figure 7: Gel electrophoresis of amplified PCR products of *hblA* (320 bp),*hblC* (750 bp), *hblD* (430 bp) genes of *B. cereus* isolates . (Number 5,9,12,13,18 represents the number ,of *B.cereus* isolates that carry two genes (*hblC* and *hblD*),and the number (11,21,41,48) represets the numbers of *B.cereus* isolates that carry one gene (*hblA*)(agarose 1.5%, TBE buffer 1X, 5V/Cm for 2.0 hrs. stained with ethidium bromide). M: The DNA molecular weight marker 100 bp ladder

The multiplex PCR technique was used to detect the presence of the *hblA*, *hblC*, *hblD*, genes of *B. cereus* (Figure 8).

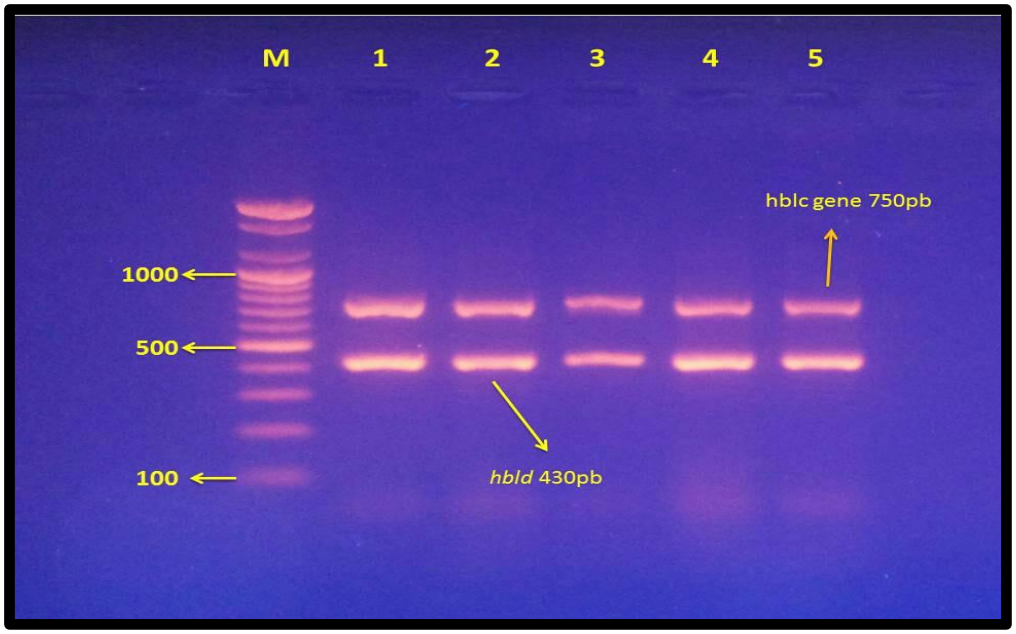

Figure 8: Gel electrophoresis of amplified PCR products of *hblC* (750 bp), *hblD* (430bp) genes of *B. cereus* isolates in multiplex PCR technique,(agarose (1.5%), TBE buffer 1X, 5V/Cm for 2.0 hrs. stained with ethidium bromide. M: The DNA molecular weight marker 100 bp ladder); Lane 1-5 a positive).

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