Investigation of toxigenic *Bacillus cereus* isolated from raw and cooked rice in Sulaimani city, KRG.

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ABSTRACT

Bacillus cereus was isolated and investigated within 100 collected samples from raw and cooked rice in Sulaimani city. Twenty-nine out of these samples were found to be contaminated with this bacterium, the highest contamination rate occurred in raw samples 60% (24/40), followed by cooked rice 8.3% (5/60). *B.cereus* densities in raw rice samples was 48.7 $\times 10^4$ CFU g−1, while in the cooked rice exceeded 10^3 CFU g−1. Identification of the twenty-two strains of the *B. cereus* was done by VITEK 2 BCL, with probability between 85%-89% which is a good and acceptable level. Then identification of this bacteria at the molecular level was done by colony PCR using primers targeting 16S rRNA gene, diarrheal toxin including non-hemolytic enterotoxin (*nheA, nheC and nheD*), hemolytic enterotoxin (*hblA, hblC, hblD*), Cytotoxin K (*cytK*), *bceT* and *entFM* enterotoxin, in addition to the emetic toxin gene (detected by colony PCR). Out of the 22 positive samples, *entFM* gene was the more abundant (95.4%.) followed by *cytK* gene which was 77.2% and the percentage of *nheA*, *nheB*, and *nheC genes* were 63.6, 13.6 and 40.9% respectively, and for *hblA*, *hblC, hblD* genes were 31.8, 22.7 and 31.8% respectively. The less percentage of these genes in these isolates was *bceT* (13.6). The detection of strains with the emetic toxin in raw and cooked rice was negative.

Keywords: *Bacillus cereus*; raw and cooked rice; toxic genes; colony PCR

1. INTRODUCTION:

Bacillus cereus is a Gram-positive bacterium, endospore-forming, biofilm producer, short rod shape, motile, grows well at 4-48 °C, optimum temperature about (28-35°C) facultative anaerobic, one of the pathogenic bacteria that cause to food poisoning (10). Spores are resistant to the adverse condition such as dry and hot, dehydration, radiation, and acidity (2). The main sources of *B. cereus* are soil and air (3). *Bacillus cereus* has been found in various natural habitat and plays a different important ecological role (4). Frequently this microorganism led to food contamination, it has been isolated from many foodstuffs such as vegetables, meat, milk and dairy product, water, pastry, noodle, and rice (5). Meanwhile, rice is one of the most popular products all over the world, and one of the major diets in many countries, that in a various way popular utilize to eat, such as frying, boiling, and cooking rice. Frequently rice contaminates due to spore of *B. cereus* during production, harvesting, handling, and processing, in which widely this microorganism exists commonly in the soil (6). Rice considers a source of vitamins, minerals, fat (2%), carbohydrate (79%), protein (7%), and pH7 and these components are suitable for growing this microorganism (7). Cooked rice left at room temperature or stored at 4-55 °C, may cause and let *B. cereus* to grow, in rice quicker than

in other food products such as beans and pasta (8). The maximum concentration of *B. cereus* spores in rice is about 10^5 CFU/g after cooking rice spores start to germinate after 24 h at 26 or 32 °C to 10^7–10^9 CFU/g (9). *Bacillus cereus* causes two types of food poisoning in humans, with 10^5-10^8 cells per gram of certain foods need to cause human disease (1). The emetic toxin which is Cereulide toxin comprises three repeats of four amino acids, (D-O-Leu-D-Ala-L-O-Val-L-Val)3 The chemical structure of the dodecadepsipeptide is closely related to the valinomycin toxin which is produced by *Streptomyces griseucauses* (10). Emesis symptoms, including, vomiting, nausea, and abnormal crump, the short onset time is about 1-6 hours after eating, and for production of this toxin *B. cereus* needs 25-30 °C (7). Emetic toxin usually heats stable and resistant to acid and trypsin. the diarrheal toxin has a long inset of about 8-16 hours (11). Typically, the foods involved include rice, pasta, and potato-based meals. *B. cereus* secrete a diarrheal toxin which is consist of five Hemolysin toxins (*hblA, hblC, hblD*) non-hemolysin (*nheA, nheC, and nheD*), cytotoxin K (*cytK*), enterotoxin T (*bceT*) and enterotoxin FM (*entFM*) (12). The minimum infective dose for the diarrheal illness caused by *B. cereus* is higher than 105 cells per gram. outbreaks of the diarrheal-illness have always been in the range of 5×10^5 to 9.5×10^8 CFU/g (7). Food typically involved comprises particularly dairy, meat, and versatile ready-to-eat foods products (10). *B. cereus* is the cause of the total cases of food poisoning for example England and Wales (0.7%), Japan (0.8%), the USA (1.3%), and Canada (2.2%). In 2018, 1.9% of total outbreaks in the European Union, with 1539 people affected with 111 hospitalizations and 1 death (13). The aim of this study is the isolation and identification of *Bacillus cereus* from raw and cooked rice, as well as the detection of diarrheal and emetic toxin gene, which cause foodborne disease.

2. MATERIALS AND METHODES:

2.1. Raw rice Samples:

Forty raw rice samples were collected from local markets in Sulaymaniyah city, 25 samples were of Kurdish seed origin and 15 were of different origin. (India, Iran, Pakistan, Uruguay, and the USA). One hundred grams of weight were taken from each sample

2.2. Cooked rice samples:

Cooked rice samples were collected from local restaurants in Sulaymaniyah city as well as homemade. Samples consisted in 60 in which 16 of those were homemade and 44 were from restaurants. The homemade cooked rice samples belong to Kurdish and Thailand seeds origin.

2.3. *Bacillus cereus* isolation:

2.3.1. Raw rice:

After taking 100g of raw rice, homogenized by a blender (Toyin/Chine), a tenfold serial dilution method was used for the cultivation of the bacteria on the Mannitol egg yolk polymyxin agar (MYP) (Liofilchem/Italy). Twenty-five grams of homogenized raw rice have been taken and mixed with 225ml peptone water (NEOGEN/UK) and six dilution tubes were placed in the water bath at 80 °C for 10min and plates were spread with each dilution and incubated for 24h at 32 °C.

2.3.2. Cooked rice:

The same procedure is repeated with cooked rice as discussed in the previous section. Microbes are preserved in slants prepared with Nutrient agar (LAB / UK) (14,15).

2.4. Identification of *B. cereus:*

2.4.1. *B. cereus* colony:

Colonies of *B. cereus* on Mannitol egg yolk Polymyxin agar (MYP) as a selective medium is large (16).

2.4.2. VITEK2 BCL compact system:

Twenty-nine samples were tested utilizing VITEK 2 BCL (BioMérieux/France), bacteria ultivated on (MYP) for 24h at 32 °C. A single colony was transmitted to 2.5 ml of sterile saline and subjected to a McFarland turbidity range of 1.80–2.20 utilizing the VITEK2 DensiChek. VITEK vacuum chamber of BCL cards was waded automatically. incubated at 35·5 °C in the VITEK 2 compact instrument and read automatically every 15min for 13h.

2.4.3. Analysis of 16S rRNA gene by Colony PCR:

For DNA extraction have been used the fresh bacterial colony which was taken into the 50µl Eppendorf tubes, colony PCR was done at 95 °C for 20min, the explicable of the 16S rRNA gene is done by colony PCR utilizing universal primers and PCR condition was Initial denaturation 94°C /5min, on step and one cycle, denaturation 95°C/30sec, annealing temperature were changed for each genes, as shown in (Table-1&2), extension 72°℃/30sec, 35 cycles, second extension 72°C/7min, final step 4℃ (17). PCR mixture for one sample consists of 5µl of PCR blue master mix (OnePCR™ Ultra/Taiwan),1µl from each primer (Macrogen/Korea), and 3µl of template DNA in a total volume of 10µl with deionized water, All PCR products were detected within 1.6% agarose gel stained with adding (5µl) ethidium bromide.

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Table 1: Primers of 16S rRNA gene.

Gene	Primers	Sequence 5-3	Tm℃	Length (bp)	Reference
16S rRNA	**16S rDNA-F**	AGAATTTGATCCTGGC TTAG	53.6	1541	(17)
	16S rDNA-R	AAGGAGGTGATCCAGC C	60.5		

2.4.4.

2.4.5. Detection of diarrheal and emetic toxin

2.4.6. gene of *Bacillus cereus:*

primers for each of the diarrheal and emetic toxin gen (Macrogen/Korea) with their product/(bp), time, and added deionized water for 100pmol/ul to each primer shown in (Table-2). To obtain the working solution, 5µl from each primer has been taken into 95µl deionized H_2O. PCR mixture for one sample consisted of 5µl master mix, 10µl deionized water, 1µl primer-F and primer-R, and 3µl sample, program cycling of the PCR shown in (Table-2)

Table 2: primers of the diarrheal and emetic toxin gene

Genes	Primers	Sequence 5-3	Tm℃	Length (bp)	Reference
Hemolytic	***hblA-F***	AAGCAATGGAATACAATG GG	54.7	1154	(11)
	hblA-R	AGAATCTAAATCATGCCACTGC	56.2		
	hblC-F	GATACYAATGTGGCAACTGC	56.4	740	
	hblC-R	TTG AGACTG CTC GYTAGT TG	57.4		
	hblD-F	ACCGGTAACACTATTCAT GC	56	829	
	hblD-R	GAGTCCATATGC TTAGATGC	53.6		
Non-hemolytic	***nheA-F***	TACGCTAAGGAGGGGCA	61	499	
	nheA-R	GTTTTTATTGCTCATCGGCT	54.7		
	nheB-F	CTATCAGCACTTATGGCAG	53.5	769	
	nheB-R	ACTCCTAGCGGTGTTCC	59.4		
	nheC-F	CGGTAGTGATTGCTGGG	58.3	581	
	nheC-R	CAGCATTCGTACTTGCCAA	56.9		
Enterotoxin FM	***entFM-F***	ATGAAAAAAGTAATTTGCAGG	50.2	1269	(11)
	entFM-R	CGTGCATCTGTTTCATGAAA	54.9		
Enterotoxin T	***bceT-F***	CGTATCGGTCGTTCACTCGG	63	924	
	bceT-R	TTTCTTTCCCGCTTGCCTTT	59.9		
Cytotoxin K	***cytK-F***	CGACGTCACAAGTTGTAACA	56.8	565	
	cytK-R	CGTGTGTAAATACCCCAGTT	56.7		
Emetic	***EMT-F***	GACAAGAGAAATTTCTACGAGCA AGTACAAT	59.5	635	
	EMT-R	GCAGCCTTCCAATTACTCCTTCTG CCACAGT	69.2		

RESULTS AND DISCUTION:

3.1. Identification by colonies and a gram stain:

A total of 100 rice samples (40 raw) and (60 cooked) were collected from local markets in Suleimani city, contaminated rice samples with *B. cereus* have been recognized with the pink colony (Mannitol negative; non-mannitol fermenter) in raw rice (60 %) (24/40) and cooked rice (8.3 %) (5/60) (Table-3). This is agreed with the (17) study, in which the highest percentage detected from raw and cooked rice was (51.5%) and (18.8 %), respectively. In cmparison with another study, the results revealed that of (48) rice samples only (13) (27.1%) samples were positive for *Bacillus cereus* (18). Navaneethan and Effarizah (19) which were isolated (34/100) *B. cereus* from cooked rice which is common. Bacteria smears have been shown short rod shape and purple color under the microscope. The high number of vegetative cells and spore total count of *B. cereus* in raw rice was about 48.7×10^4 CFU/g and 4×10^3CFU/g. This is not agreed with Sarrıas *et al*., (20) in which raw rice samples did not reach 10^2 CFU/g. In addition to cooked rice samples the high number of the vegetative total count was about

2.52 $\times 10^{-3}$CFU/g as shown in (Table-4). This is agreed with (Jessim *et* al., (13) in which samples of cooked rice have fewer numbers of bacteria except one sample had a record 4.9×10^{3} CFU/g.

Table3: *B. cereus* total count from raw (R*) and cooked rice (C*) samples

Samples No. (R*)	Vegetative total count/g	spore's total count/g
R. 1	3.2×10^{2}	
R.2	3.6×10^{2}	-
R.3	3×10^{3}	-
R.4	3×10^{3}	-
R.5	4×10^{3}	
R.6	3.3×10^{2}	2.69×10^{2}
R.7	3.1×10^{2}	
R.8	3.2×10^{2}	
R.9	4×10^{3}	
R.10	2.5×10^{2}	
R.11	3.3×10^{2}	
R.12	48.7×10^{4}	2.5×10^{2}
R.13	5.2×10^{2}	4×10^{2}
R.14	3.2×10^{2}	2.5×10^{2}
R.15	7.3×10^{2}	3.1×10^{2}
R.16	6×10^{3}	-
R.17	4.6×10^{2}	-
Samples No. (C*)	**Vegetative cell total count**	
C.1	2.5×10^{2}	
C.2	4×10^{3}	
C.3	3×10^{3}	
C.4	4×10^{3}	
C.5	7×10^{3}	

3.2.

3.3. Identification of *B. cereus* by VITEK 2 BCL system and 16S rRNA gene:

VITEK 2 BCL has been utilized to recognize *Bacillus cereus*, VITEK compacts system is faster than manual techniques for identification (22) (Table-5) results were probability between 89%-85% which is a good and acceptable level. Identification of *Bacillus cereus* from positive samples was done by VITEK 2 BCL, results were different for raw rice due to a change in percentage from (24/40) (60%) to (17/40) (42%), while cooked rice samples were the same (5/60) 8.3% (Table-5). Moreover, all 14 strains of *Bacillus cereus* were identified by VIT2 BCL which were 100% (21). the results of Vitek 2 system divided in to probability of very good identification= 93-95% Probability of good identification= 89-92%, Probability of acceptable identification =85-88. Five positive cooked and 17 positive raw rice samples were subjected to identification by the16S rDNA molecular method. Furthermore, reported in 20 samples from each local

indigenous and imported rice samples, were detected by PCR analysis 100% of the local indigenous and 85% of the imported rice grain were positive (23).

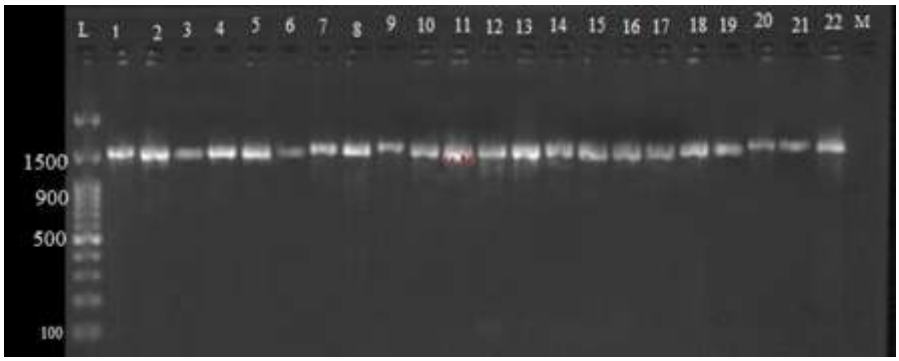


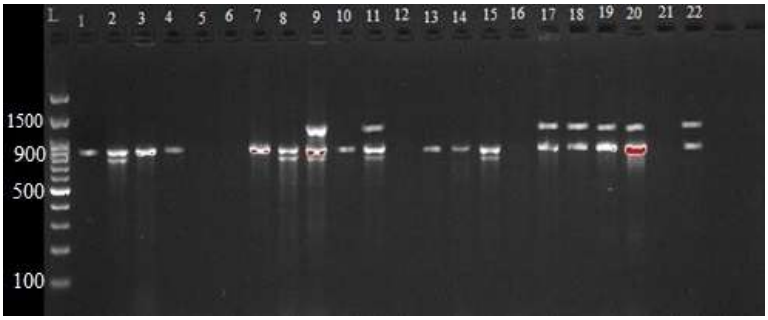
Figure (1). Identification of *Bacillus cereus* by detection 16S rDNA. Lane (L)= Ladder

Lanes (1,2,3,4,5,6,7,11,12,13,14,15,16,17,18,19,21 (raw rice), Lanes (8,9,10,20,22) (cooked rice) were positive isolates (1541bp), M= Negative control.

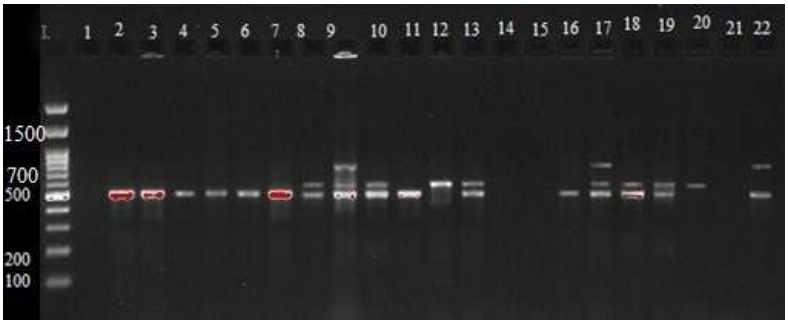
3.4. Detection of diarrheal genes:

3.4.1 Detection of (*hbl* and *nhe*) genes:

A total of 22 samples of positive diarrheal genes included *nheA, nheB, nheC* (63.6%, 13.6% and 40.9%), respectively and *hblA, hblC, hblD* (31.8%, 22.7%, and 31.8%) followed by *entFM, bceT* and *cytK* (95.4%, 13.6%, and 77.2%). Each positive sample of *Bacillus cereus* isolated from raw and cooked rice was tested for diarrheal genes such as hemolytic (*hblA, hblC, hblD)* and non-hemolytic (*nheA, nheB, nheC)* by colony PCR. In seventeen raw rice of *hblD*, and *nheA* was found highest percentage which was (82.3%) (14/17). respectively. while *nheB* was found lowest percentage (5.88%) (1/17). Followed by *nheC*, *hblA*, *hblC*, were found (35.2%) (6/17), (23.5%) (4/17), and (17.6%) (3/17), and in five cooked rice samples, the genes of the *nheA* and *hblD*, *nheC* had the highest percentage (80%) (4/5), (60%) (3/5), and the lowest percentage was found concerning *nheB. hblC* and *hblA* (40%) (2/5). (Table-5&6, Figure-2&3)



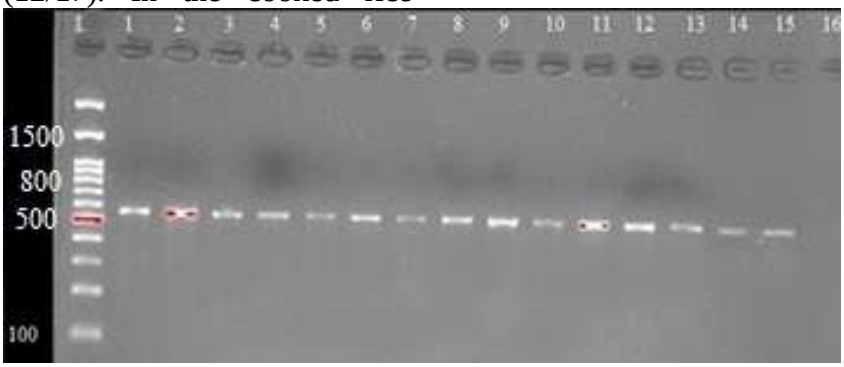
Figure(2). Colony PCR of *hblA, hblC, hblD* genes of *Bacillus cereus* Isolates. Lane (L)= Ladder, Lanes 11, 17,18 and 20 (raw rice). Lanes 9 and 22 (cooked rice), Samples were positive for *hblA*(1154)bp, Lanes 2,11,15 (raw rice), lanes 8,9(cooked rice) were positive for *hblC*(829)bp, Lanes 1,2,3,4,7,11,13,14,15,17,18,19,20,22 (raw rice). Lanes 8,9,10 (cooked rice) were positive for *hblD*(740)bp,



Figuer(3). Colony PCR of *nheA, nheB,nheC* genes of *Bacillus cereus* isolates. Lane (L)=Ladder, Lanes 2,3,4,5,6,7,8,11,13,16,17,18,19 (raw rice) Lanes 8,9,10,22 (cooked rice) were positive for *nheA* (499) bp Lanes 17(raw rice), Lanes 9,22 (cooked rice) were positive for *nheB*(769)bp, Lanes. 12,13,17,18,19, 20 (Raw rice), Lanes 8,9,10 (cooked rice) were positive for, *nheC*(581)bp

3.4.2. Detection of cytotoxin K (*cytK*):

In raw rice was found (70.5%) (12/17). In the cooked rice isolate, the gene was found in (60%) (3/5), and (Table-5&6, Figure-4)



Figure(4). Colony PCR for detection cytK genes of *Bacillus cereus* isolates. Lane (L)= Ladder, Lanes. Raw rice (1,2,3,4,5,6,7,11,12,13,14,15) cooked rice (8,9,10) were positive for cytK (565) bp

3.4.3. Detection of the *entFM* gene:

Only one sample from raw rice was negative for this gene, the highest percentage was in raw rice (100%) (17/17) cooked rice (80%) (4/5). (Table-5&6, Figure-5)

Figure(5). Colony PCR for detection entFM genes of *Bacillus cereus* isolates. Lane (L)= Ladder, Lanes. 1-19 (Raw rice). Lanes 8,9,10,21 (cooked rice) were positive for *entFM* (1269).

3.4.4. Detection of *bceT* gene:

Highest percentage were found in cooked rice (60%) (2/5), and in raw rice (5.8%) (1/17) (Table-5&6, figure-5). This is agreed with a previous study in which *B. cereus* genes were detected in *hblC* (49%) and *nheA* (89%) respectively as well as, and all strains of *B. cereus* were positive for the present *entFM* gene while *cytK* gene was found in 68% of the obtained isolates (9). Although, *hblC*, *hblD*, and *nheC* genes were detected in cooked rice samples and had the highest percentage (40%) followed by *hblA*, *nheA*, and *nheB* genes in which formed (20%) *entFM* (0%), cytK (100%), and *bceT* (100%), as well as in uncooked rice *hblA* (20%), *hblC* (70%), and *hblD* (*50%)* followed by *nheA (*20%) *nheB* (60%), and *nheC* (50%), for each of the *entFM* (0%), *cytK* (90%), *bceT* (90%) (11). In another study, shown that *nheA* carriers were the most prevalent (26/29), followed by *hblA* (21/29) (24). However, research published, in which 81.4% of isolates obtained from rice were able to produce *nhe*, 57.6% *hbl* (25). Withal, has been 47 (56.6%) detected *hblA* and *hblD* genes were detected and 74 (89.1%) detected the *nheA* and *nheB* genes (26).

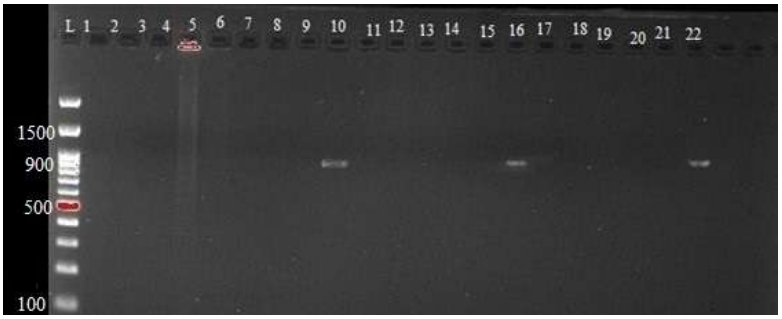


Figure (5). Colony PCR for detection enterotoxin genes *bceT* of *Bacillus cereus* isolates.

Lane (L)= Ladder, Lanes 16 (raw rice), Lanes 10,22 (cooked rice) were positive for *bceT* (924))bp

3.5. Detection of emetic toxin gene:

3.5.1. Detection of EMT gene:

This gene wasn`t found in each of the rice sample isolates (Table-6) this is agreed with the study, the emetic gene was not detected in raw and cooked rice samples (17,26). However, research published in Iraq by Aubaid *et al*., (27) conducted that 18 isolated *Bacillus cereus* in cooked rice (50%) were positive for the existence of emetic toxin gene. Also, *B. cereus* isolated from (37) raw rice only one sample contained emetic toxin (28). Whereas, the predominant of *Bacillus cereus* emetic strains in the habitants mostly very low reference by Delbrassinne *et al*., (29) in which emetic cereulide toxin was detected 7.4% of rice dishs from various restaurants in China. emetic B. cereus was detected (5/29) (24). Moreover, reported by Samapundo *et al.* (26) in which emetic toxin encoding genes (ces) were detected (6.8%).

Table4: Analysis of 16s rDNA, diarrheal and emetic gene of the Bacillus cereus from raw rice

Sample`s No. (R)	16S rDNA	*entFM*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*bceT*	*cytK*	*EMT*
R.1	+	+	+	-	+	-	-	+	-	-	-
R.2	+	+	+	-	+	+	-	+	-	-	-
R.3	+	+	-	-	+	+	-	-	-	+	-
R.4	+	+	-	-	+	+	-	-	-	+	-
R.5	+	+	-	-	+	-	-	-	-	+	-
R.6	+	+	-	+	+	+	-	-	-	+	-
R.7	+	+	-	-	-	+	-	-	-	+	-
R.8	+	+	+	+	+	-	-	-	-	+	-
R.9	+	+	-	-	-	+	-	-	-	+	-
R.10	+	+	-	+	+	-	-	-	-	+	-
R.11	+	+	-	-	-	+	-	-	+	-	-
R.12	+	+	+	-	+	+	+	+	-	-	-
R.13	+	+	+	-	+	+	-	+	-	-	-
R.14	+	+	-	-	+	+	-	-	-	-	-
R.15	+	+	-	-	+	-	-	-	-	+	-
R.16	+	+	-	-	-	-	-	+	-	+	-
R.17	+	+	-	-	+	+	-	+	-	+	-

3.6. Acknowledge:

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