Detection of the *hbl* complex genes in *Bacillus cereus* isolated from cow raw milk in northwest of Iran

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Abstract

Bacillus(B) cereus is regarded as a major foodborne pathogen which is widely distributed in the nature. In addition, it plays an important role in the contamination of ready-to-eat and dairy products. *B. cereus* causes the two different types of food poisoning in human: the diarrheal and the emetic type. The aim of this study is detection of *hbl* complex genes in *B. cereus* isolated from cow raw milk in Northwest of Iran. In the present study, the number of the samples collected from cow raw milk were 120. All the isolates already had been identified phenotypically, and they were assessed for molecular confirmation by using the PCR method. *B. cereus* isolates were determined by detecting the *hbl* genes complex in the isolates. The result of this study showed that *B. cereus* were found in the raw milk samples 117 (97.5%) from the 120 samples. The frequency of the *hbl*A, *hbl*C, and *hbl*D genes found in *B. cereus* isolates were 105 (89.7%), 102 (87.1%), and 102 (87.1%) , respectively. 99 isolates (84.6%) harboured 3 tested genes simultaneously. 12 *B. cereus* isolates (10.3%) lacked these genes. The results of current study showed that *B. cereus* isolated from raw milk have high potential in causing food poisoning and therefore the use of the procedures to reduce the bacterial contamination during the processing of dairy product is required.

***Keywords*:** *Bacillus cereus*, raw milk, *hbl* genes complex

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الكشف عن معقد الجينات *hb1* في جراثيم العصويات الشمعية المعزولة من حليب البقر الخام في شمال غرب إيران

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الخلاصة

تعتبر جرثومة العصويات الشمعية من مسببات الامراض الرئيسية التي تنتقل عن طريق الغذاء والمنتشرة على نطاق واسع في الطبيعة. بالإضافة الى ذلك، فانها تلعب دورا مهما في تلوث منتجات الالبان الجاهزة للأكل. تسبب العصيات الشمعية نوعين مختلفين من التسمم الغذائي للإنسان: نوع الاسهال ونوع القيء. الهدف من هذه الدراسة هو الكشف عن مركب الجينات *hbl* في العصيات الشمعية المعزولة من حليب البقر الخام في شمال غرب ايران. جمعت ١٢٠ عينة من حليب الابقار الخام. تم التعرف على جميع العزلات من خلال الصفات المظهرية، وكذلك تم تقييم العزلات للتأكيد الجزيئي باستخدام طريقة تفاعل البلمرة المتسلسل. تم تحديد عزلات العصيات الشمعية بالكشف عن مركب الجينات *hbl* في العزلات. اظهرت نتائج الدراسة العثور على العصيات الشمعية في ١١٧ (٩٧,٥%) عينة من عينات الحليب الخام من مجموع ١٢٠ عينة. تم ايجاد جينات *hbl* A، *hbl* C و *hbl* D في العصيات الشمعية ١٠٥ (٨٩,٧%)، ١٠٢ (٨٧,١%) و ١٠٢ (٨٧,١%)، على التوالي. كذلك اظهرت الدراسة ان ٩٩ (٨٤,٦%) من العصيات الشمعية تحتوي على ثلاث جينات، بينما اكدت الدراسة ان ١٢ (١٠,٣%) من العصيات الشمعية لا تحتوي على مركب الجينات *hbl*. اظهرت نتائج الدراسة الحالية ان

العصيات الشمعية المعزولة من الحليب الخام لها القدرة الكبيرة في إحداث التسمم الغذائي لذلك فان اتباع الاجراءات الصحية المناسبة يقلل من التلوث بالجراثيم خلال مراحل تصنيع منتجات الالبان.

Introduction

Foodborne disease is regarded as a one of the most important disease which causes a serious problem in developed and developing countries (1). Raw milk is considered a good medium for growth and proliferation of the algae, protozoans, fungi, bacteria, and viruses because it has the most important nutrition factors. There are many types of pathogenic bacteria have been isolated from raw milk, some of these pathogenic bacteria are able to form spores and can tolerate the pasteurization conditions. *B. cereus* is one of the most important pathogens that tolerates the pasteurization process (2). This bacterium is usually a source of raw milk contamination and a major microbiological problem in the dairy industry. The heat resistant of the *B. cereus* spores is a source of contamination for milk products (3). *B. cereus* has many pathogenicity factors which causes diarrhea associated with production of enterotoxins such as the hemolysin BL (*hbl*), nonheliolateral enterotoxin (NHE), cytotoxin K, and FM enterotoxin (4). *B. cereus* produces the toxin in the small intestine that causes food poisoning and diarrhea (5). The hemolysin BL toxin is consisting of a three-component protein complex (6), which is formed from a sticky component (B) and two lithic components (1L and 2L), that coded the *hblA*, *hblD*, and *hblC* genes, respectively. The presence of the three genes are necessary for maximum activity and poisoning (7). The *B. cereus* infectious dose which causes the food poisoning is 10^4-10^{11} cells per 1 gram of food. The exact amount of toxin depends on the several factors, such as presence of vegetative bacterial cells, sporulated form in food, amount of produced enterotoxins, and the sensitivity of target cell population (8-10). The aim of this study was to detect the *hbl* complex genes in *B. cereus* isolated from raw cow's milk in Northwest of Iran.

Materials and methods

Samples collection

In this study, 300 cow raw milk samples collected from different regions in northwest of Iran (the period of collect the samples was from April to October in 2018). All the samples were tested by using the culture and biochemical tests to detect the characteristics of *B. cereus* isolates and finally, 120 *B. cereus* isolates were identified and they were sent to molecular identification by using PCR method.

Molecular detection of *B. cereus* and the *hbl* complex genes

DNA extraction of the *B. cereus* isolates was performed by using the DNA extraction kit (Pak Gene Yakhteh company, Iran). The quality of extracted DNA samples was evaluated by using the Nano Drop instrument and suitable samples to select for the next steps. The *B. cereus* specific primers used in the present study (Nano Zist Fanavaran company, Iran) (Table 1).

The PCR reaction was performed in a total volume of 20 µl containing 10×PCR buffer 2 µl, $MgCl_2$ 2 mM, dNTP 0.2mM, specific primers (0.25 µM), Taq DNA polymerase 1.5 U, and extracted DNA 4 µl using the thermal cycler (Astec, Japan). The PCR conditions for each gene are presented in the Table 2. The obtained PCR products were electrophoresed on 1.5% agarose gel (11).

Results

The result of this study decleared that *B. cereus* found in the 117 samples from the 120 investigated samples which were previously detected by using biochemical tests, and they were confirmed as *B. cereus* by using PCR reaction (Figure 1).

Table 1: Sequence of primers used for detection of *B. cereus* and the *hbl* complex genes

Gene	Sequence (5′-3′)	Amplicon size	Reference
Bal	F: 5′-TGCAACTGTATTAGCACAAGCT-3′ R: 5′-TACCACGAAGTTTGTTCACTACT-3′	533 bp	9
hblA	F: 5′-GTGCAGATGTTGATGCCGAT-3′ R: 5′-ATGCCACTGCGTGGACATAT-3′	320 bp	10
hblC	F: 5′-AATGGTCATCGGAACTCTAT-3′ R: 5′-CTCGCTGTTCTGCTGTTAAT-3′	750 bp	10
hblD	F: 5′-AATCAAGAGCTGTCACGAAT-3′ R: 5′-CACCAATTGACCATGCTAAT-3′	430 bp	10

Table 2: The thermocycler programs for detection of *B. cereus* and *hbl* complex genes

Primer	Temperature (ºC)/Time(sec/min)					Cycle
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	
Bal	94ºC (03:00)	94ºC (00:30)	54ºC (00:45)	72ºC (01:00)	72ºC (05:00)	35
hblA	94ºC (04:00)	94ºC (00:30)	58ºC (00:45)	72ºC (01:00)	72ºC (05:00)	35
hblC	94ºC (03:00)	94ºC (00:30)	53ºC (00:45)	72ºC (01:00)	72ºC (05:00)	35
hblD	94ºC (04:00)	94ºC (00:30)	54ºC (00:45)	72ºC (01:00)	72ºC (05:00)	35

The Figure 2 showed that the rate of the *hblA* gene found in the *B. cereus* isolates was 89.7% (105/117). In addition, the Figure 3 appeared that the rate of the *hblC* gene found in the *B. cereus* isolates was 87.1% (102/117). Moreover, the Figure 4 declared that the rate of the *hblD* gene found in the *B. cereus* isolates was 87.1% (102/117). Also, all the three genes were detected in the *B. cereus* isolates 99 (84.6%). In the other hands, 12 isolates of *B. cereus* (10.25%) were without studied genes (Table 3).

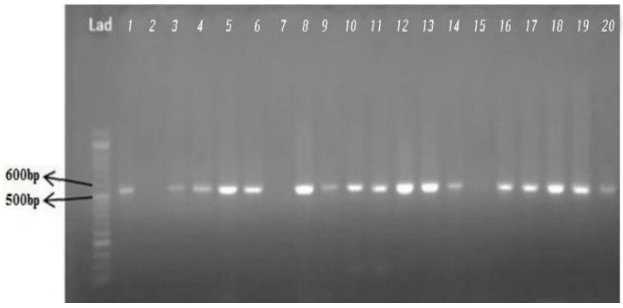

Figure 1. Electrophoresis of the *bal* gene PCR product on 1.5% agarose. Lad: ladder 50 bp; No. 1: positive control (*B. cereus* ATCC 11778); No. 2: negative control (double-distilled water); No. 3-6 and 8-14 and 16-20: positive *B. cereus* samples; No. 7 and 15: negative *B. cereus* sample.

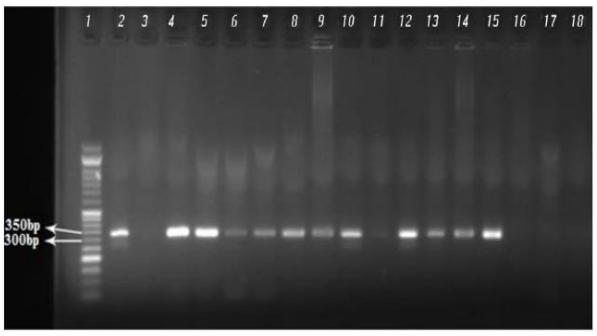

Figure 2. Electrophoresis of the *balA* gene PCR product on 1.5% agarose. No. 1: ladder 50 bp; No. 2: positive control (*B. cereus* ATCC 11778); No. 3: negative control (double-distilled water); No. 4-15: positive *B. cereus* samples; No. 16-18: negative *B. cereus* sample.

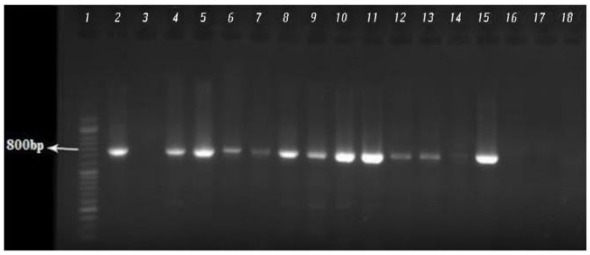

Figure 3. Electrophoresis of the *balC* gene PCR product on 1.5% agarose. No. 1: ladder 50 bp; No. 2: positive control (*B. cereus* ATCC 11778); No. 3: negative control (double-distilled water); No. 4-15: positive *B. cereus* samples; No. 16-18: negative *B. cereus* sample.

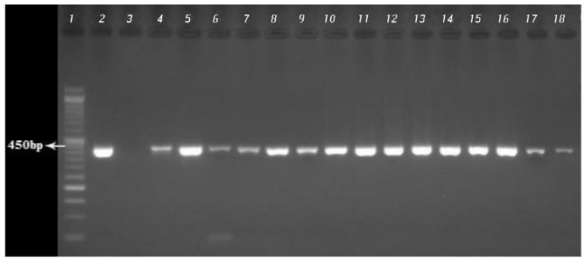

Figure 4. Electrophoresis of the *balD* gene PCR product on 1.5% agarose. No. 1: ladder 50 bp; No. 2: positive control (*B. cereus* ATCC 11778); No. 3: negative control (double-distilled water); No. 4-18: positive *B. cereus* samples.

Table 3. Frequency of *hbl* complex genes in studied isolates

Genes	Isolates number	Frequency (%)
hblA	105	89.7%
hblC	102	87.1%
hblD	102	87.1%
hblA + hblC	102	87.1%
hblA + hblD	102	87.1%
hblC + hblD	99	84.6%
hblA+hblC+hblD	99	84.6%

Discussion

In present study, 120 cow raw milk samples collected from different regions in Northwest in Iran. All the *B.*

cereus isolates were previously detected by using the phenotypic culture and the biochemical tests. After PCR reaction by using the specific primers, 117 *B. cereus* isolates were detected as a *B. cereus*, genetically. This indicates a higher accuracy of PCR method than the culture biochemical tests. The rapid methods for identify presence of enterotoxigenic *B. cereus* in foods is very important to ensure the foods hygiene. The culture and Biochemical tests are less accurate compared with the PCR reaction, which is more accurate and more reliable. In the present study, the frequency of *hblA*, *hblC* and *hblD* genes were showed 105 (89.7%), 102 (87.1%) and 102 (87.1%), respectively. In the previously study by Kim *et al*. (12) in South Korea reported that the prevalence of *hblA* and *hblC* genes in standard strains of *B*. *cereus* were 6.25%, and the frequency of *hblD* gene was 25%. In another study showed that only 12.5% of the isolates had all the three genes, simultaneously (12), which is much less than frequency of mentioned genes in present study. Deilami and Nasiri (13) reported that the frequency of the *hbl* complex genes in *B. cereus* isolated from foodstuffs in Tabriz and Zanjan restaurants was 8%, which is also much less than frequency of mentioned genes in present study. Prub *et al.* (14) reported that the prevalence of the *hblA* gene in *B. cereus* was 43%. Reis *et al*. (15) reported that 36.5% of isolated *B. cereus* from pasteurized, sterilized and dry milk in Brazil had the *hbl* complex genes. In another study, El-zamkan and Mubarak in Egypt (16), has been reported that the frequency of the *hbl* complex genes in *B. cereus* isolated from ice cream and rice-milk was 33.3% and 43.5%, respectively (16). Differences in distribution of the *hbl* complex genes in different *B. cereus* isolates in the mentioned studies probably are due to the geographical differences and the differences in ecological origin of isolated strains from milk, rice, meat, salads. Due to presence of all the three *hbl* complex genes simultaneously in studied *B. cereus* isolates in this study, the hemolysin BL enterotoxin will have its maximum activity, and these isolates will potentially be highly pathogenic, if *hbl* complex genes are expressed. Many factors affect the microbial quality of raw milk, which four factors considered as main sources in microbial contamination of raw milk. These resources include inside of livestock breast, exterior of livestock breast, environmental factors, and milking equipment and maintenance. Therefore, in order to provide hygienic milk and its products, health care must be respected according to Hazard Analysis and Critical Control Point (HACCP) instructions, during the production and consumption (2,17). In general, the culture method and the biochemical tests are time-consuming and less accurate than the PCR method. Using the PCR test, in addition to being quicker, has more accuracy and confidence.

Conclusion

In this study, regarding that the most of the tested *Bacillus cereus* isolates harboured all the three *hbl* genes, in the case of the expression of these genes, these isolates will have high virulence potentially.

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Conflict of interest

The authors declare that they have no conflict of interest.

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