Detection of the \textit{hbl} complex genes in \textit{Bacillus cereus} isolated from cow raw milk in northwest of Iran

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Abstract

\textit{Bacillus}(\textit{B}) \textit{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. \textit{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 \textit{hbl} complex genes in \textit{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. \textit{B. cereus} isolates were determined by detecting the \textit{hbl} genes complex in the isolates. The result of this study showed that \textit{B. cereus} were found in the raw milk samples 117 (97.5\%) from the 120 samples. The frequency of the \textit{hblA, hblC, and hblD} genes found in \textit{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 \textit{B. cereus} isolates (10.3\%) lacked these genes. The results of current study showed that \textit{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: \textit{Bacillus cereus}, raw milk, \textit{hbl} genes complex

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الكشف عن معقد الجينات \textit{hbl} في جراثيم العصويات الشمية المعزولة من حليب البقر الخام في شمال غرب إيران

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الخلاصة

تعتبر جراثيم العصويات الشمية من مسببات الأمراض الرئيسية التي تنتقل عن طريق الغذاء والانتشار على نطاق واسع في الطبيعة. بالإضافة إلى ذلك، فإنها تلعب دورا مهما في تلوث منتجات الألبان الجاهزة للأكل. تسبب العصويات الشمية نوعين مختلفين من التسمم الغذائي للإنسان: نوع الأسماك ونوع الفم. الهدف من هذه الدراسة هو الكشف عن معقد الجينات \textit{hbl} في العصويات الشمية المعزولة من حليب البقر الخام في شمال غرب إيران. جمعت 120 عينة من حليب البقر الخام. تمت التعرف على جميع العزلات من خلال الصفات المظهرية، وكذلك تم تقييم العزلات للتثبيت المجهري باستخدام طريقة تفاعل البلمرة المتسلسل. تم تحديد عزلات العصويات الشمية بالكشف عن معقد الجينات \textit{hbl} في العزلات. أظهرت نتائج الدراسة العثور على العصويات الشمية في 117 (97.5\%) عينة من عزلات الحليب السائل من مجموع 120 عينة. تم إيجاد جينات \textit{hbl D} و \textit{hbl C} و \textit{hbl A} في العصويات الشمية 105 (87.1\%) عينة. و في العصويات الشمية 102 (87.1\%) عينة. بينما اكتمل الدراسة أن 99 (87.1\%) من العصويات الشمية تحتوي على ثلاث جينات، بينما اكدت الدراسة أن 12 (10\%) من العصويات الشمية لا تحتوي على معقد الجينات \textit{hbl}.
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 10⁴-10¹¹ 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, MgCl₂ 2 mM, dNTP 0.2mM, specific primers (0.25 µM), Taq DNA polymerase 0.2U, 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′<br>R: 5′-TACCACTACGCTTTCATCT-3′ | 533 bp | 9 |
| hblA | F: 5′-GTGCAGATGGGATCGCGAT-3′<br>R: 5′-ATGCCACTGTCTGGGACATAT-3′ | 320 bp | 10 |
| hblC | F: 5′-AATGGTCATCGGAACTCTAT-3′<br>R: 5′-TCGCTTGTCTGCTTGAAT-3′ | 750 bp | 10 |
| hblD | F: 5′-AATCAAGAGGCTGTCAGAAT-3′<br>R: 5′-CACCAATTGACCAGCTAAT-3′ | 430 bp | 10 |
Table 2: The thermocycler programs for detection of B. cereus and hbl complex genes

| Primer | Initial denaturation | Denaturation | Annealing | Extension | Final extension | Cycle |
|--------|----------------------|--------------|-----------|-----------|-----------------|-------|
| Bal    | 94°C (03:00)         | 94°C (00:30) | 54°C (00:45) | 72°C (01:00) | 72°C (05:00) | 35    |
| hblA   | 94°C (04:00)         | 94°C (00:30) | 58°C (00:45) | 72°C (01:00) | 72°C (05:00) | 35    |
| hblC   | 94°C (03:00)         | 94°C (00:30) | 53°C (00:45) | 72°C (01:00) | 72°C (05:00) | 35    |
| hblD   | 94°C (04:00)         | 94°C (00:30) | 54°C (00:45) | 72°C (01:00) | 72°C (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](image1.png)

**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](image2.png)

**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](image3.png)

**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](image4.png)

**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](image5.png)

**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.
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 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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