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OPTIMIZATION OF DNA EXTRACTION TO DETECT *BACILLUS CEREUS* FROM FOOD USING A PCR TECHNIQUE - SHORT COMMUNICATION

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Bacillus species are extremely widespread in nature and may be isolated from soil, water, dust, air and many foods. The genus *Bacillus* is characterized by the formation of heat resistant endospores. *B. cereus* has been recently considered as an 'emerging' food-borne pathogen. A couple of new primers (BCFW1 and Bcrevnew) directed to identify *B. cereus* is described as well as protocols for optimization of DNA extraction for basic groups of food products which are particularly prone to contain this microorganism. To avoid inhibitory effects exerted by particular food matrices, specific pre-extraction steps are recommended in this work: a treatment with α-amylase from *Aspergillus oryzae* E.C. 3.2.1.1. (Sigma, Germany) for boiled rice or washing the pellet with EDTA solution for minced meat, pasteurized milk and salads. The lowest possible level of detection was obtained for pasteurized milk (20 cells/g) and salad (30 cells/g) and it was comparable with the results of classical microbiological analyses. Generally, for milk, salad and rice samples the limit of detection was close to 50 cells/g, whereas for minced meat it was definitively higher (about 500 cells/g) and did not reflect results of performed standard plate counts. As the result of the studies, promising, handy protocols for (i) an efficient DNA extraction from food matrices and (ii) fast, species-specific PCR identification were prepared and presented.

INTRODUCTION

Many species of the spore-forming genus *Bacillus* could be found in foods, such as meat products, dairy products, vegetables and rice. *Bacillus cereus* has been identified as the causative agent in a number of food poisoning outbreaks. The illness has been attributed to the presence of enterotoxins and other toxins including haemolysins produced by strains of *B. cereus*. There is still confusion regarding how many different enterotoxins are produced by *B. cereus*, in fact *B. cereus* produces a high variety of toxins and enzymes that are active on different cell tissues that has been recently associated with the capability of this microorganism to induce non-gastrointestinal infections as systemic and pulmonary infections [Ghelardi *et al.*, 2000]. Both types of food poisoning (due to the production of either an emetic toxin or diarrhoeal toxins) are usually caused by heat-treated foods, where surviving spores are the source of food poisoning [Granum *et al.*, 1996]. However, reports of *B. cereus* poisoning are few, because not much attention has been paid and many cases go underdetected [Kramer & Gilbert, 1989].

Conventionally, *B. cereus* is detected by its ability to grow on selective plating media containing egg yolk and inability to utilize mannitol. The isolates are identified by morphological, cultural and biochemical characteristics. Because a desirable approach is to detect organisms directly in the food, the aim of this work was to develop a PCR technique useful to detect *B. cereus* in foods and to improve the recovery of a low number of *B. cereus* from contaminated foods also in the presence of other contaminating microorganisms.

MATERIALS AND METHODS

Artificial contamination of food products. *B. cereus* DSMZ 2301 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was grown overnight onto PCA (Oxoid) plates, and then suspended in sterile distilled water. Different *B. cereus* concentrations ranging from 1×10^6 cells/mL to 1-10 cells/mL were prepared using the Bürker camera. The real estimation of the inocula was made by plating 0.1 mL of each sample dilution onto *Bacillus cereus* Selective Agar base (BCSAB, Oxoid) immediately after the inoculation. Dry rice (300 g) was added to 1 L of boiling distilled water in a sterile 2-L glass beaker, boiled for 15 min and cooled to room temperature. Samples of minced meat (10 g), boiled rice and salad were inoculated with 1 mL of different concentrations of *B. cereus*, the 1 mL of pasteurized milk was inoculated with 0.1 mL of the different concentrations of *B. cereus*. As described above, a part of each inoculated sample after dilution was used to verify the *B. cereus* concentration by plating onto BCSAB (Oxoid). After 48-h incubation at 30°C, a viable count was done and values were expressed in CFU/g.

DNA extraction from bacteria. Bacteria isolated from BCSAB (Oxoid) were grown overnight at 30°C on Brain Heart Infusion (BHI) agar (Oxoid, Milan, Italy) and a single colony was picked from the plate and transferred into a 1.5 mL tube containing 0.3 g glass beads and DNA extraction was performed as described by Manzano *et al.* [2003]. DNA concentration was standardized at about 50 ng/μL before being used in the PCR assay.

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DNA extraction from food samples

Rice. 10 grams of rice were homogenized for 2 min in a Stomacher 80 (PBI, Milan, Italy) in 90 mL of diluent after the addition of the *B. cereus* solution. Subsampling of 10 mL was used for DNA extraction. To remove any PCR inhibitory substances from food samples, the 10 mL homogenates obtained were centrifuged at 20 000 rpm at 10°C for 10 min. The pellet was resuspended in 1.5 mL solution of α-amylase from *Aspergillus oryzae* E.C. 3.2.1.1 (Sigma A-6211) and after 10 min was centrifuged at 14 000 rpm at 10°C for 10 min and washed two times in 1.5 mL sterile distilled water.

Meat. 10 grams of minced meat were homogenized for 2 min in a Stomacher 80 (PBI, Milan, Italy) in 90 mL of diluent after the addition of the *B. cereus* solution. Subsampling of 10 mL was used for DNA extraction. To remove any PCR inhibitory substances from food samples, the 10 mL homogenates obtained were centrifuged at 20 000 rpm at 10°C for 10 min. The pellet was resuspended in 1.5 mL of EDTA 0.25 M for 10 min and centrifuged at 14 000 rpm for 10 min and then washed two times in 1.5 mL sterile distilled water.

Milk. 1 mL of milk was added to 8 mL of diluent and after the addition of 1 mL of the *B. cereus* solution was homogenized for 2 min by vortex and used for DNA extraction. To remove any PCR inhibitory substances from food samples, the 10 mL homogenates obtained were centrifuged at 20 000 rpm at 10°C for 10 min. The pellet was resuspended in 1.5 mL of EDTA 0.25 M for 10 min and centrifuged at 14 000 rpm for 10 min and then washed two times in 1.5 mL sterile distilled water.

Salad. 10 grams of salad were homogenized for 2 min in a Stomacher 80 (PBI, Milan, Italy) in 9 mL of diluent after the addition of the *B. cereus* solution. Subsampling of 1 mL was used for DNA extraction. To remove any PCR inhibitory substances from food samples, the 1 mL homogenate obtained was centrifuged at 10 000 rpm at 4°C for 5 min. The pellet was resuspended in 0.5 mL of EDTA 0.25 M for 10 min and centrifuged at 10 000 rpm for 5 min at 4°C and then washed two times in 0.5 mL sterile distilled water.

The pellets obtained were used for DNA extraction using glass beads as reported by Manzano *et al*. [2003]. DNA samples were stored at -20°C until being used. To obtain reliable results (error-prone due to complexity of food matrices and its PCR inhibitory properties), experiments were repeated at least three times each.

PCR conditions. Two primers, designed BCFW1 and BCrevnew (Figure 1) for the amplification of the *gyrB* gene of the *Bacillus* species were used. Primers were designed by authors using *Amplify* software (Madison, WI, USA). DNA sequences were retrieved from the GenBank (AF 090330, AF 136387, AF 136388 for *B. cereus*, AF 090332 for *B. mycoides*, AF 090331 and AF 36390 for *B. thuringiensis*). PCR assays were performed in a reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 200 µM each dNTP, 0.2 µM each primer, 3.5 mM $MgCl_2$, 1.25 U Taq Gold Polymerase (Applera Italia, Monza, Italy), 2 µL DNA (50 ng/µL) in a final volume of 50 µL.

Thermal Cycler conditions consisted of 95°C denaturation for 5 min, 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min and a final extension at 72°C for 7 min in a Mini--Cycler (MJ Research, M-Medical Genenco, Florence, Italy).

PCR fragments were analyzed by submarine gel electrophoresis [Sambrook *et al.,* 1989], stained with ethidium bromide, (0.5 µg/mL) and then visualized under UV light and photographed using a Syngene Cabinet (Cambridge, UK).

RESULTS

The high specificity shown by the couple of primers used in this study towards the *B. cereus* (no amplification products were obtained for other microrganisms) allowed to use them for the selective detection of *B. cereus* strains directly in foods. On the basis of the different composition of the food matrices, it was necessary to prepare different pre-extraction methods for the different foods analyzed to obtain DNA amplification using the PCR technique.

Boiled rice: As shown in Figure 2, lane 7, the treatment with α-amylase enabled to obtain a specific amplicon for the minimum level of 50 cells/g.

Minced meat: In this case washing the pellet with EDTA solution was beneficial. This protocol enabled to obtain specific amplification products for the minimum level of 5×10^2

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                    10        20        30        40        50        60
                    |         |         |         |         |         |
Bcereus             CATGCAGGAGGCAAATTTGACGGTGGCGGTTATAAAGTTTCTGGTGGTTTACATGGTGTT
Bthuringiensis      CATGCTGGTGGGAAATTCGACGGTGGCGGTTATAAAGTTTCTGGTGGTTTGCACGGTGTT
Bmycoides           CATGCTGGTGGTAAGTTTGACGGTGGTGGTTATAAAGTTTCTGGTGGTTTGCATGGTGTA
Consensus           CATGCtGGtGG AAaTTtGACGGTGGcGGTTATAAAGTTTCTGGTGGTTTgCAtGGTGTt
Prim.cons.          CATGCTGGTGG3AAATTTGACGGTGGCGGTTATAAAGTTTCTGGTGGTTTGCATGGTGTT

                    70        80        90        100       110       120
                    |         |         |         |         |         |
Bcereus             GGGGCATCGGTTGTAAATGCTCTATCAACAGAATTAGAAGTATTTGTACATCGTGAAGGT
Bthuringiensis      GGTGCATCTGTTGTAAATGCCTTATCAACAGAATTAGAAGTATTTGTACATCGTGATGGC
Bmycoides           GGTGCATCTGTTGTAAATGCTTTATCAACAGAATTAGAGGTATTTGTACATCGAGATGGA
Consensus           GGtGCATCtGTTGTAAATGCttTATCAACAGAATTAGAaGTATTTGTACATCGtGAtGG
Prim.cons.          GGTGCATCTGTTGTAAATGCTTTATCAACAGAATTAGAAGTATTTGTACATCGTGATGG3

                    130       140       150       160       170       180
                    |         |         |         |         |         |
Bcereus             AAAATCCATTACCAAAAATACGAAAGAGGTATTCCGGTTGCGGATTTAAAAGTCATTGGT
Bthuringiensis      AAAATCCATTACCAAAAATACCAAAGAGGTATTCCGGTTGCAGATTTAAAAGTCATCGGT
Bmycoides           AAAATACACTATCAAAAATATGAACGCGGGGTCCCGGCTGCGGATTTAAGAGTAATAGGT
Consensus           AAAATcCAtTAcCAAAAATAcgAAaGaGGtaTtCCGGtTGCgGATTTAAaAGTcAT GGT
Prim.cons.          AAAATCCATTACCAAAAATACGAAAGAGGTATTCCGGTTGCGGATTTAAAAGTCAT3GGT

                    190       200       210       220       230       240
                    |         |         |         |         |         |
Bcereus             GACACCGATCAAACAGGAACAATAACTCGATTTAAACCAGATCCGGAAATTTTCCAAGAA
Bthuringiensis      GATACAGATAAGACTGGAACAATAACTCGCTTTAAACCGGATCCAGAAATTTTTAAAGAG
Bmycoides           GAAACTGATCGTACAGGAACGATCACACGCTTTAAGCCAGATTCAGAAATTTTTACAGAG
Consensus           GA AC GATca ACaGGAACaATaACtCGcTTTAAaCCaGATcCaGAAATTTTtaaAGAg
Prim.cons.          GA3AC3GATCA3ACAGGAACAATAACTCGCTTTAAACCAGATCCAGAAATTTTTAAAGAG

                    250       260       270       280       290       300
                    |         |         |         |         |         |
Bcereus             ACAACAGTATACGATTTTGATACGCTAGCAACTCGTATGCGTGAATTAGCGTTTTTAAAT
Bthuringiensis      ACGACAGAATATGAATTCGATACGCTCGCGACTCGTGTGCGTGAGTTGGCGTTTTTAAAT
Bmycoides           ACGACAGAATACGAATTTGATACATTAGCGACTCGTATGCGTGAGTTGGCGTTTTTAAAT
Consensus           ACgACAGaATAcGAaTTtGATACgcTaGCgACTCGTaTGCGTGAgTTgGCGTTTTTAAAT
Prim.cons.          ACGACAGAATACGAATTTGATACGCTAGCGACTCGTATGCGTGAGTTGGCGTTTTTAAAT

                    310       320       330       340       350       360
                    |         |         |         |         |         |
Bcereus             CGTAATATTAAATTAACAATTGAAGATAAACGTGAACGTAAGCAAAAGAAAGAATTCCAT
Bthuringiensis      CGTAATATTAAATTAACAATTGAAGATAAACGTGAACATAAGCAAAAGAAAGAGTTCCAC
Bmycoides           CGTAATATTAAATTAACAATTGAAGATAAGCGTGAACATAAGCAAAAGAAAGAATTCCAT
Consensus           CGTAATATTAAATTAACAATTGAAGATAAaCGTGAACaTAAGCAAAAGAAAGAaTTCCAt
Prim.cons.          CGTAATATTAAATTAACAATTGAAGATAAACGTGAACATAAGCAAAAGAAAGAATTCCAT

                    370       380       390       400       410       420
                    |         |         |         |         |         |
Bcereus             TACGAAGGTGGAATTAAATCATACGTTGAGCATTTAAATCGCTCAAAACAACCGATTCAT
Bthuringiensis      TATGAAGGTGGAATTAAATCATATGTTGAACATTTAAATCGTTCAAAACAACCAATTCAT
Bmycoides           TATGAAGGTGGAATTAAATCATATGTTGAACATTTAAATCGTTCAAAACAACCGATTCAT
Consensus           TAtGAAGGTGGAATTAAATCATAtGTTGAaCATTTAAATCGtTCAAAACAACCgATTCAT
Prim.cons.          TATGAAGGTGGAATTAAATCATATGTTGAACATTTAAATCGTTCAAAACAACCGATTCAT
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FIGURE 1. Allignement of *Bacillus cereus* (AF090330), *B. mycoides* (AF090332) and *B. thuringiensis* (AF090331) *gyr B* gene sequences (Multalin program, version 5.4.1). Underlined are the primers BCFW1 (forward:5' gtttctggtggtttacatgg3') and BCrevnew (reverse: 5' ttttgagcgatttaaatgc3'), PCR product of 374 bp.

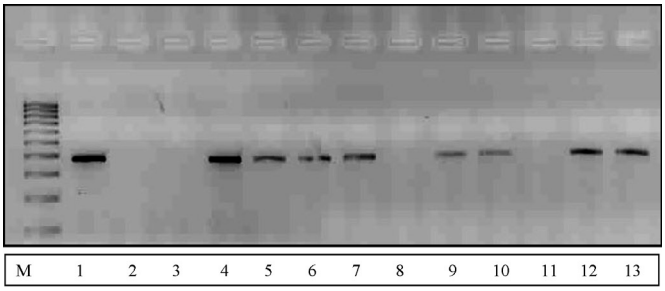

FIGURE 2. PCR products obtained for artificially contaminated food samples (374 bp). M: Molecular Weight Marker (100 bp, Sigma, Germany); Lane 1: *Bacillus cereus* DSMZ2301; Lane 2: negative control; Lane 3: boiled rice not artificially contaminated; Lane 4: boiled rice contaminated with 1×10^3 cells/g; Lane 5: boiled rice contaminated with 1×10^2 cells/g; Lane 6: boiled rice contaminated with 50 cells/g; Lane 7: salad not artificially contaminated; Lane 8: salad not artificially contaminated (no *B. cereus*): Lane 9: milk artificially contaminated with 1×10^2 cells/g; Lane 10: milk contaminated with 20 cells/g; Lane 11: milk not artificially contaminated; Lane 12: minced meat artificially contaminated with 5×10^2 cells/g; Lane 13: minced meat artificially contaminated with 1×10^4 cells/g.

cell/g of *B. cereus* using only 2 mL of DNA extracted (Figure 2, lane 13).

Pasteurized milk: For the DNA extraction from milk it was better to clean the pellet using EDTA solution, and an amplicon was achieved down to the level of 20 cells/g of *B. cereus* (Figure 2, lane 11).

Salads: The specific amplification product for *B. cereus* was still obtained at the level of 30 cells/g of *B. cereus* cells, that is the lowest number detectable also using the classical microbiological analyses, due to the dilution employed in the technique used (Figure 2, lane 8).

DISCUSSION

The detection limit of *B. cereus* directly from the food samples gave different results because the matrix of the food affected the detection capability of the microbial cells using the PCR technique. As well-known, sometimes extracting microbial DNA directly from food, Taq polymerase inhibitory substances present in the sample could decrease the efficiency of the amplification reaction.

In case of salads, the most naturally contaminated samples, the presence of natural contaminating microflora did not hinder with the amplification of the selected specific DNA target. It was possible to detect 30 cells/g of *B. cereus* of sample, a value very close to the lower limit of detection obtained for the milk (which was not diluted before the DNA extraction) and corresponding to 20 cells mL^{-1}. For the milk, salad and rice sample the values obtained were about of the same range, within the 50 cells/mL or g, while for minced meat the level was definitely higher, at 5×10^2 cells/g. In case of minced meat, it was not possible to obtain the detection of *B. cereus* at the same level as during the classical microbiological analyses (Table 1), while for the other samples used the lowest detectable limit was the same and was established based on the dilution obtained from the homogenization of the sample.

TABLE 1. Viable counts of food products used in this study (values expressed in CFU/g or L).

Sample	Mesophilic microflora	Yeasts and moulds	*Bacillus cereus*
Boiled rice	<30	<30	<30
Milk	<10	<10	<10
Salads	1.6×10^6	2×10^4	30
Minced meat	1.3×10^8	<30	<30

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