J Ind Microbiol Biotechnol (2004) 31: 161–169
DOI 10.1007/s10295-004-0125-x

ORIGINAL PAPER

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Detection of *Bacillus cereus* group bacteria from cardboard and paper with real-time PCR

Received: 7 July 2003 / Accepted: 15 February 2004 / Published online: 3 April 2004

Abstract The aim of this study was to develop a PCR-based rapid method to detect *Bacillus cereus* group cells from paper and cardboard. Primers targeting the 16S rDNA and real-time PCR with SYBR green I detection were used in order to be able to also quantify the target. Both autoclaved cardboard samples spiked with *B. cereus* vegetative cells or spores and naturally contaminated paper and cardboard samples were studied. Results were compared with culturing verified by commercial (API) tests. Several different methods were tested for DNA isolation from the paper and cardboard samples. Two commercial kits intended for soils, the UltraClean soil DNA kit and the FastDNA spin kit for soil, gave the most reproducible results. In spiked samples, the average yield was 50% of added vegetative cells, but spore yield was only about 10%. PCR results from adding vegetative cells correlated with added colony-forming unit (cfu) values ($r = 0.93$, $P < 0.001$) in the range 100–10,000 cfu g^{-1}. Three out of nine studied paper and cardboard samples contained *B. cereus* group bacteria, based both on culturing and real-time PCR. The numbers were 10^2–10^3 bacteria g^{-1}; and PCR gave somewhat higher results than culturing. Thus, real-time PCR can be used as a rapid semi-quantitative method to screen paper and cardboard samples for contamination with *B. cereus* group bacteria.

Keywords *Bacillus cereus* · Packaging · Hygiene · SYBR green I · Endospores · Food

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Introduction

The genus *Bacillus* is a diverse collection of saprophytic bacteria widely distributed in the environment, especially in soil and wood. They are unified by endospore formation, which makes them resistant to drying, heat and other extreme conditions. Certain *Bacillus* species may cause problems in the paper and packaging industry. Some, e.g. *B. subtilis*, spoil raw materials, whereas others produce slime, which disturbs the process and causes defects in the end-products. The species *B. cereus* is pathogenic and causes two types of food-borne diseases: diarrhea and emesis. Apart from the emetic toxin (cereulide), at least three different enterotoxins are produced by *B. cereus* [20]. *B. cereus* has been isolated from a variety of food samples, such as pasteurized milk, dairy products, rice, noodles, spices, bread, fast foods and vegetarian foods [1, 17, 36, 37, 42]. There are strains of *B. cereus* which have been shown to cause food poisoning at a very low infectious level (10^3–10^4 bacteria g^{-1}) [2]. *B. cereus* group species (*B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*) share a high degree of chromosomal sequence similarity; and all of them, excluding *B. anthracis*, have been shown to be potentially toxigenic, with the potential to cause foodborne illness outbreaks and opportunistic infections [8, 14, 21, 25, 26, 35].

The main contaminants in food-packaging paper and board belong to the genera *Bacillus* and *Paenibacillus* [34, 40, 41, 44]. Pirttijärvi et al. [34] studied liquid-packaging boards and characterized over 200 isolates. Contaminants found were aerobic spore-forming bacteria, among which the *B. cereus* group was often present. Approximately 50% of the *B. cereus* group strains were positive in enterotoxin tests. Even if no such cases have, to our knowledge, been reported, *Bacillus* spores present in packaging materials for food might in some circumstances contaminate foods, by being in contact with them for prolonged storage times. Suominen et al.

[41] showed that the interface between the paperboard and its polymer coating facing the food could be a potential site from which microbes could leak into the food. Manufacturers of packaging materials for food have become increasingly aware of customer demands relating to concerns of food safety; and they are expected to bring their hygiene practices in line with the expectations of the food industry. In addition to food packaging, packaging for hygienic and medical products have high purity demands.

Traditionally, *B. cereus* has been identified from homogenized samples by culturing isolates on selective media. Most of these methods are based on the inability of *B. cereus* to use mannitol as a carbon source and its ability to produce lecithinase, which causes the degradation of egg yolk. In addition, polymyxin is usually included in the media, since *B. cereus* is not affected by concentrations of polymyxin which inhibit the microbiota commonly accompanying it. Even though selective media are used, typical colonies must be confirmed by morphological and biochemical characters [microscopy, API tests (bioMérieux) or, more recently, by molecular biological methods, e.g. ribotyping [4], RAPD-typing [4] or sequencing the 16S rRNA gene [19]. Culturing *B. cereus* on selective media takes 2 days, so with additional tests the detection time is several days.

PCR methods have also been used to detect *B. cereus*, e.g. using primers targeting the 16S rRNA gene [22], the major cold-shock protein genes [18], the neutral protease gene [7] or the toxin genes [6, 25, 32] of *B. cereus*. However, most studies have been done on pure cultures or spiked samples. PCR is very sensitive, but sample preparation of different matrices often presents problems. If very low bacterial levels must be detected, samples need to be homogenized and concentrated. *B. cereus* spores have a high hydrophobicity, a low spore-surface charge and their surfaces are covered by adherence-aiding appendages [3], which can cause problems in sample homogenization due to their tight adherence to surfaces. In addition, when cells are concentrated, potentially PCR-inhibitory substances are also concentrated. Thus, purification of DNA is usually necessary. The clear advantage of PCR is that detection times are much shorter than in culture-based detection. As far as we know, there is no published method for the PCR detection of bacteria directly from paper products.

In conventional PCR, where end-point detection is used, quantification of the target organism is not possible. In real-time PCR, amplification is monitored continuously during the PCR reaction, which permits quantification of the target, based on the fact that the more target there is present, the earlier amplification starts. There are several options for detection of the product during amplification, e.g. hybridization probes, Taq Man probes or SYBR green I dye. The probes enable sequence-specific recognition of the target, whereas SYBR green I is an intercalating dye which binds to the minor groove of double-stranded DNA and, upon binding, increases its fluorescence over 100-fold. Therefore, SYBR green I detects all double-stranded DNA, including non-specific products. Real-time PCR equipment, however, enables the determination of melting curves for the amplification products at the end of the run, to verify the specificity of the product.

The aim of this study was to develop a rapid method for detecting *B. cereus* group bacteria from paper and cardboard samples. DNA isolation from a large sample volume by concentrating it through centrifugation or filtration was compared with isolation using four commercial kits. In order to determine also the number of target bacteria, real-time PCR with SYBR green I chemistry was used. Both spiked cardboard samples and naturally contaminated samples were studied. Results were compared with culturing on selective media and verification of the colonies by API tests.

Materials and methods

Bacterial strains

The bacterial strains used in this study were obtained from the VTT culture collection [39] and are listed in Table 1. The strains were cultured aerobically in nutrient broth (NB) or on nutrient agar (NA; Difco Laboratories, Detroit, Mich.). Strains were incubated overnight at 30 °C, except strain E-95571^T (27 °C), strain E-97206^T (25 °C) and strain E-81128^T (55 °C).

Preparation of spores

Spores of *B. cereus* were prepared in order to use them for testing the DNA extraction methods. *B. cereus* E-96680 was incubated at 30 °C for 7 days on NA supplemented with 10 mg l^{-1} $MnSO_4$. Bacterial suspensions were then heat-shocked for 15 min at 80 °C, cooled on ice and centrifuged (10,000 *g*, 10 min). Pellets were resuspended in 0.25 strength Ringer's solution (Merck, Darmstadt, Germany). The success of spore preparation was confirmed microscopically with Schaeffer–Fulton spore-staining [38]. Spores were stored at −20 °C until used.

Cardboard and paper samples

Nine samples were obtained from paper mills and included various paperboard raw materials and end-products. In some experiments, samples were sterilized by autoclaving them and spiking them with bacteria. Either a culture of *B. cereus* E-96680 grown overnight in NB at 30 °C or frozen spores were used. Serial 10-fold dilutions of the suspensions were added to homogenized cardboard and paper suspensions. The cell densities of the *B. cereus* cell or spore suspensions were determined afterwards by plating onto Plate count agar (Difco Laboratories, Detroit, Mich.) and incubating the plates

Table 1 Bacterial strains used in this study. ^{T} Type strain

Species	Strain number	Origin	Species	Strain number	Origin
Bacillus cereus	E-93143^{T}	DSM 31^{T} (ATCC 14579^{T})	*B. licheniformis*	E-95571^{T}	DSM 13^{T} (ATCC 14580^{T})
B. cereus	E-95600	Recycled fiber board	*B. licheniformis*	E-95602	Recycled paper board
B. cereus	E-96717	Polyethylene-coated paper board	*B. pumilus*	E-95572^{T}	DSM 27^{T} (ATCC 7061^{T})
B. cereus	E-97747	Pea-protein product	*B. pumilus*	E-991364	End-product from paper mill
B. cereus	E-98968	Pasteurized paper machine slime	*B. pumilus*	E-991370	Raw material paper
B. cereus	E-96680	Recycled fiber pulp sample	*B. simplex*	E-95604	Recycled paper board
B. cereus	E-97816	Pasteurized milk	*B. sphaericus*	E-95580	Recycled paper board
B. cereus	E-96681	Process water of a mill	*B. stearothermophilus*	E-81128^{T}	DSM 22 (ATCC 12980)
B. cereus	E-96682	Recycled fiber board	*B. subtilis*	E-97009^{T}	DSM 10 (ATCC 6051)
B. cereus	E-981023	Paper mill	*B. subtilis*	E-991361	End-product from paper mill
B. cereus	E-991373	Raw material paper	*B. subtilis*	E-95601	Recycled paper board
B. cereus -group	E-96677	Recycled paper board	*B. subtilis*	E-96715	Polyethylene-coated paper board
B. mycoides	E-97206^{T}	DSM 2048^{T} (ATCC 6462^{T})	*Citrobacter freundii*	E-96692	Recycled pulp
B. thuringiensis	E-86245^{T}	DSM 2046^{T} (ATCC 10792^{T})	*Enterobacter* sp.	E-991307	Paper sack for foodstuffs
B. thuringiensis	E-95624	Salad	*Pseudomonas putida*	E-92005	Soil

at 30 °C for 1–2 days. For spores, heat-activation (80 °C, 10 min) and incubation in L-alanine buffer (10 mM L-alanine, 50 mM Tris-HCl, pH 7) at 37 °C for 1 h were also tried before adding them to the samples.

Detecting *B. cereus* from cardboard samples by culturing

Three grams of cardboard was aseptically cut into pieces 1–2 cm^2 and homogenized with 297 ml of a solution of 0.85% NaCl, 1% peptone in a Waring Blender (Waring Commercial, Torrington, Conn.) for 2 min at low speed. Then, 10 ml of the resulting suspension was heat-shocked in a water-bath (80 °C, 15 min). Dilution series were prepared from both non-shocked and shocked suspensions and surface-spread (0.1 ml) on Mossel plates (*Cereus*-selective agar base according to Mossel; Merck, Darmstadt, Germany) or PEMBA plates (*B. cereus* selective agar base; Oxoid, Basingstoke, UK) plates. Mossel plates were incubated at 30 °C for 2 days and PEMBA plates at 37 °C for 1 day and then at room temperature for 1 day. Typical colonies were further tested by the API 50 CHB/E test (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

PCR sample preparation from pure cultures

Template DNA for PCR analysis was prepared from cultures (Table 1) grown overnight in NB. For testing the specificity of primers, cells were harvested from 0.5–1.0 ml of culture by centrifugation (13,000 *g*, 3 min). The pellet was resuspended in a suspension of 500 µl of glass beads (acid-washed, 150–212 µm diam.; Sigma Chemical Co., St Louis, Mo.) in sterile H_2O (1:6). The cells were then mechanically disrupted in a FastPrep FP120 (Savant Instruments, Holbrook, N.Y.) at 6.5 m s^{-1} for 30 s. Later, for standard solutions, dilutions of the pure cultures were made in sterile H_2O as follows: 0.1 g of autoclaved glass beads were added to 0.5 ml of cell suspension and cells were disrupted from each dilution separately, using a Vibrogen cell mill (Edmund Bühler, Johanna Otto, Hechingen, Germany) for 3 min. Glass beads and cell debris were removed by centrifugation (13,000 *g*, 3 min) and the supernatant was used directly as a template in the PCR reaction.

Cell-disruption experiments

To test the adequacy of a 2-min lysis, different concentrations of both *B. cereus* E-96680 spores and vegetative cells were treated otherwise as described above, but kept in the cell mill for 0, 2, 3, 4, 5 or 10 min. The suspensions were used directly as templates in real-time PCR reactions to compare which disruption time gave the highest amplification rate. A 3-min lysis was shown to release DNA from both vegetative cells and spores equally efficiently; and longer lysis times did not give higher yields (data not shown). In addition, the disruption efficiencies of the FastPrep and Vibrogen cell mills were tested and shown to be equal (data not shown).

PCR sample preparation from cardboard samples

For cardboard samples, several different methods to isolate DNA were tested. First, a modification of the homogenization technique used in culturing was applied. Five grams of cardboard samples were cut into pieces approximately 1–2 cm^2 and homogenized in a Waring Blender with 200 ml of 0.85% NaCl, 1% peptone solution for 2 min. To remove the solids, the suspension was filtered with vacuum suction, using a Büchner funnel and Whatman No. 1 filter paper, and the volume of the remaining liquid was measured. The cells were then concentrated by centrifugation (4,000–10,000 *g*, 20–30 min) and resuspended in 0.5 ml of H_2O. Concentrating samples by filtration through a

polycarbonate filter (Isopore HTTP, 0.4 μm, 47 mm diam.; Millipore, Billerica, Mass.) or a Teflon filter (Fluoropore FHLP, 0.4 μm, 47 mm diam.; Millipore) was also attempted, but the filters were clogged by the solution. The cells were then mechanically disrupted using a Vibrogen cell mill for 3 min. Glass beads and cell debris were removed by centrifugation (13,000 *g*, 3 min) and the supernatant was used as a template in the PCR reaction, either directly or after purification with a High Pure PCR template preparation kit (Roche Diagnostics, Penzberg, Germany). Purification was done according to the manufacturer's instructions, except that 300 μl of supernatant was used, the cell lysis steps were omitted and 50 μl of elution buffer was used.

Four commercial DNA isolation kits were also tried: the UltraClean soil DNA kit and the UltraClean soil DNA kit Mega prep from MoBio Laboratories (Solana Beach, Calif.) and the FastDNA spin kit for soil and the FastDNA kit from Qbiogene (Carlsbad, Calif.). In three kits, subsamples of cardboard (0.1 g) were cut into pieces of approximately 0.5 cm^2 and added to the lysis tubes, whereas 2 g of cardboard were used in the MoBio Mega kit. DNA isolation was performed according to the manufacturers' instructions, except that cells were mechanically disrupted for 3 min in a FastPrep mill, the centrifugation steps were longer and the elution volumes were 100 μl. Also, in spore-addition experiments, longer disruption times (up to 10 min) were tested. When the MoBio Mega kit was used, cardboard was homogenized in a Waring blender for 4 min, using the solution supplied in the kit, the resulting suspension was transferred to the bead tube of the kit, disrupted in the Vibrogen cell mill for 3 min and isolated DNA was concentrated by isopropanol precipitation.

PCR primers

Two different primer pairs specific for *B. cereus* group cells were tested with the strains listed in Table 1. The target of the first primer pair (forward primer 5′ CTT (C/T)TT GGC CTT CTT CTA A 3′, reverse primer 5′ GAG ATT TAA ATG AGC TGT AA 3′) was the major cold-shock protein gene of *B. cereus*, *B. thuringiensis* and *B. mycoides* [18]. The target of the second primer pair (forward primer 5′ TCG AAA TTG AAA GGC GGC 3′, reverse primer 5′ GGT GCC AGC TTA TTC AAC 3′') was 16S rDNA; and this pair also amplified *B. anthracis*, *B. pseudomycoides* and *B. weihenstephanensis* [22]. The sizes of the PCR products of the first and second primer pairs were 284 bp and 288 bp, respectively. The primers were purchased from Sigma-Genosys (Cambridge, UK) and had been purified by desalting them.

Real-time PCR

The LightCycler PCR machine (Roche Diagnostics, Penzberg, Germany) was used. For initial experiments and for testing the primer specificity and sensitivity with pure cultures, the following conditions (based on preliminary experiments) were used. Reactions were performed in a 20-μl reaction volume with 2 μl of LightCycler–FastStart DNA master SYBR green I (Roche), containing dNTPs, polymerase enzyme, buffer and SYBR green I fluorescent dye. The final $MgCl_2$ concentration was 4 mM and the primer concentration was 1 μM for both primer pairs. The amount of template was either 2 μl (major cold-shock protein gene primers) or 5 μl (16S rDNA primers). The temperature program for the major cold-shock protein genes was: 10 min initial denaturation at 95 °C, followed by 45 cycles of 15 s denaturation at 95 °C, 5 s annealing at 55 °C, and 12 s elongation at 72 °C, at the end of which the fluorescence was measured. For the 16S rDNA primers, the reaction conditions were the same, except that the annealing temperature was 63 °C.

Later, only primers targeting 16S rDNA were used. When cardboard samples were analyzed, further optimization of the PCR reaction became necessary. The PCR reaction was optimized to get the maximum amount of product and minimum amount of primer dimers. In addition, reaction efficiency was monitored from the slope of the standard curve. Different $MgCl_2$ concentrations (1–5 mM), primer concentrations (0.2–1.0 μM), annealing temperatures (63–65 °C) and annealing times (3–5 s) were tested. In addition, fluorescence was measured at different temperatures (72–84 °C). The optimized reaction conditions were 4 mM $MgCl_2$ and 0.3 μM primers. The temperature program was: 10 min initial denaturation at 95 °C, followed by 45 cycles of 15 s denaturation at 95 °C, 5 s annealing at 63 °C, 12 s elongation at 72 °C and 1 s at 84 °C, during which the fluorescence data were acquired. All PCR reactions were performed with two to three replicates. Both H_2O and DNA isolated from cardboard sterilized by autoclaving were always included as negative controls.

PCR standards

To test the linearity and the dynamic range of the real-time PCR reaction, three dilution series of *B. cereus* E-96680 and one dilution series each of *B. cereus* DSM 31^T, *B. mycoides* DSM 2048^T and *B. thuringiensis* DSM 2046^T were compared.

Five dilutions of standards were included in each LightCycler run, ranging over approximately 1–10,000 cfu per PCR reaction. All results were calculated using an external standard curve, which was an average of 22 measurements of a dilution series of *B. cereus* E-96680. The same fluorescence noise level (0.2) and threshold level (0.2) were always used for determination of crossing-points (i.e. the cycle in which the fluorescence signal crosses the threshold level) of the standards and samples.

Statistical analyses

For standard curves, regression analysis was performed to determine the slope and coefficient of determination. The Pearson correlation coefficient was calculated for comparing cfu and PCR results from spiked samples. The analyses were performed with SPSS software (SPSS Inc., Chicago, Ill.).

Results

Both primer pairs were specific for *B. cereus* group cells (Table 2). The 16S rDNA primers were chosen for further use, since the 16S rRNA gene is a structural gene which is not as prone to variation as protein genes and more sequence information is available for 16S rDNA.

In all PCR reactions, the melt curves of the products were checked, in order to verify that a correct amplification product was formed. The melting temperature of the product was 88.7 ± 0.5 °C for pure cultures and samples prepared with the MoBio kit and was 87.7 ± 0.5 °C for samples prepared with the FastDNA kit. Non-specific amplification (primer dimers) was detected at temperatures of 77–78 °C. In cardboard samples, the formation of both the specific product and an unspecific product was sometimes observed. In spite of the optimization experiments, this phenomenon could not be totally prevented. Nevertheless, since fluorescence was measured at 84 °C, non-specific products were excluded from the result, although they might have affected amplification efficiency.

The crossing-points from real-time PCR using dilution series of *B. cereus* E-96680, *B. cereus* DSM 31^T, *B. mycoides* DSM 2048^T and *B. thuringiensis* DSM 2046^T were linear in the range 1–10,000 cfu per PCR reaction (Fig. 1A). Higher dilutions were not tested. The quantitative detection limit of the PCR reaction was set to crossing-point 31, which corresponds to approximately 1 cfu per PCR reaction, because after that the results were not reproducible.

The type strains of *B. cereus* and *B. thuringiensis* had lower crossing-points than *B. cereus* E-96680 and the type strain of *B. mycoides* (Fig. 1A). Real-time PCR crossing-points were linear and reproducible both when the same standards were measured several times (Fig. 1B) and when several different dilution series were measured from the same bacterial strain (Fig. 1C). The coefficient of determination (r^2) from regression analyses was, respectively, 0.992 and 0.991 for the same dilution series and for different dilution series. The average efficiency ($\eta = 10^{(-1/\text{slope})} - 1$) of the PCR reaction in standard curves was 90%. The average coefficient of variation between replicate PCR reactions in the same run was 1.1% and the average coefficient of variation for the same templates in different runs was 1.5%.

Real-time PCR results from spiked cardboard samples where DNA was isolated with Waring Blender homogenization and centrifugation did not give reproducible results, nor did DNA isolation with the MoBio Mega kit or the FastDNA kit (data not shown). Reproducible results were obtained when DNA was isolated with the MoBio or FastDNA for soil kits. The results from PCR correlated statistically significantly ($r = 0.93$, $P < 0.001$) with cfu values in the range 100–10,000 cfu g^{-1} cardboard (Fig. 2). MoBio and FastDNA kits did not differ significantly in their DNA isolation efficiency. Results from PCR were, on average, 50% of the added cfu values. Nevertheless, when spores were added, the yield was repeatedly only about 10% (data not shown). Extending the cell disruption time to 10 min and activation of spores by heat-treatment or L-alanine before adding them did not increase the yield.

Three of the nine cardboard and paper samples studied contained 10^2–10^3 *B. cereus* group cells g^{-1}, detected both with culturing and real-time PCR (Table 3). Also, in real-time PCR, samples 1–5 pro-

Table 2 Comparison of amplification results in real-time PCR with various bacterial strains and two different primer pairs specific for the *B. cereus*group. *PCR^A* Primers targeting the major cold shock protein gene, *PCR^B* primers targeting the 16S rDNA gene. + Specific product, # non-specific product, – no product

Strain	PCR^A	PCR^B	Strain	PCR^A	PCR^B
B. cereus E-93143^T	+	+	*B. licheniformis* E-95602	–	#
B. cereus E-95600	+	+	*B. licheniformis* E-95571^T	–	#
B. cereus E-96717	+	+	*B. pumilus* E-991364	#	–
B. cereus E-97747	+	+	*B. pumilus* E-95572^T	#	#
B. cereus E-98968	+	+	*B. pumilus* E-991370	#	–
B. cereus E-96680	+	+	*B. simplex* E-95604	#	–
B. cereus E-97816	#	+	*B. sphaericus* E-95580	–	–
B. cereus E-96681	+	+	*B. stearothermophilus* E-81128^T	–	–
B. cereus E-96682	+	+	*B. subtilis* E-991361	–	#
B. cereus E-981023	+	+	*B. subtilis* E-95601	–	#
B. cereus E-991373	+	+	*B. subtilis* E-96715	–	#
B. cereus group E-96677	+	+	*B. subtilis* E-97009^T	–	#
B. mycoides E-97206^T	+	+	*Citrobacter freundii* E-96692	–	#
B. thuringiensis E-95624	+	+	*Enterobacter* sp. E-991307	#	#
B. thuringiensis E-86245^T	+	+	*Pseudomonas putida* E-92005	–	–

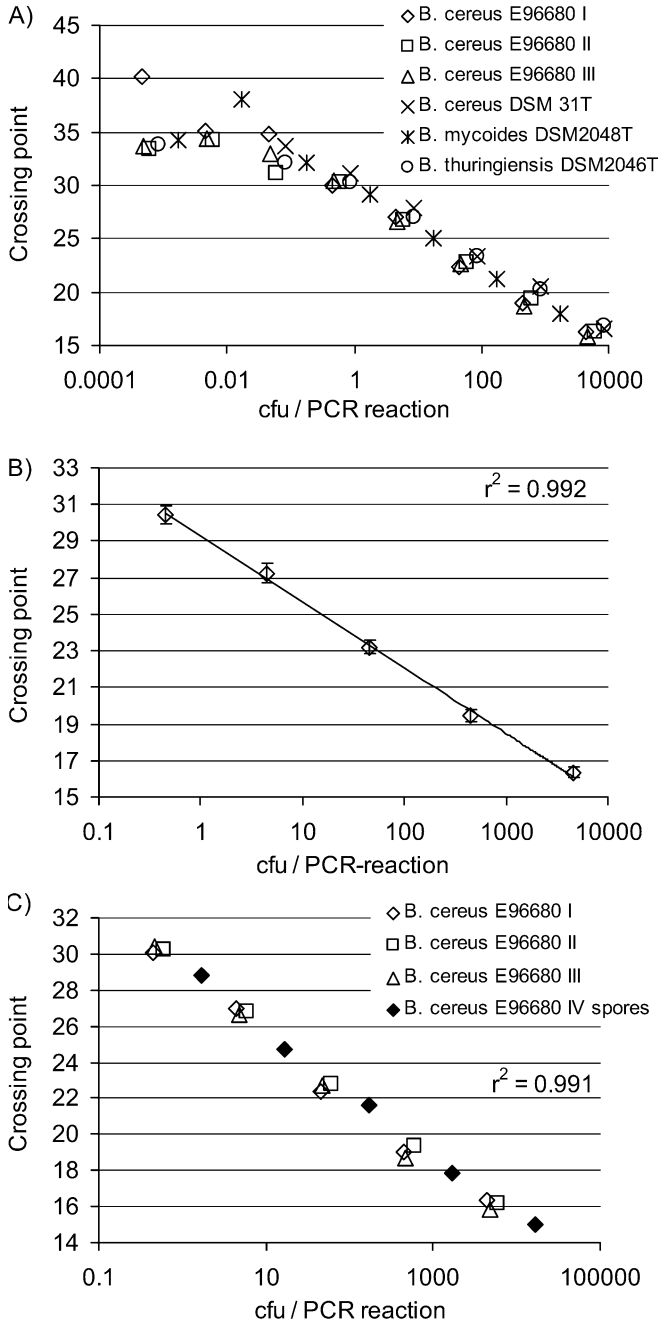

Fig. 1A–C Real-time PCR crossing-points plotted versus colony-forming unit (*cfu*) counts per PCR reaction. Sample DNA was isolated from dilution series of pure cultures. **A** Results from three separate dilution series of *Bacillus cereus* strain E-96680 and dilution series of *B. cereus*, *B. mycoides* and *B. thuringiensis* type strains. **B** One dilution series of *B. cereus* E-96680. The results shown are means for 22 separate measurements. *Bars* Standard deviations. **C** Four different dilution series of *B. cereus* E-96680, three from vegetative cells and one from spores

duced a specific amplification product, but only after cycle 31, indicating that low numbers of *B. cereus* group cells were present. The FastDNA kit gave somewhat higher results than the MoBio kit. Plate counts were performed twice, but results are given as a range of variation, since identification of colonies by API tests

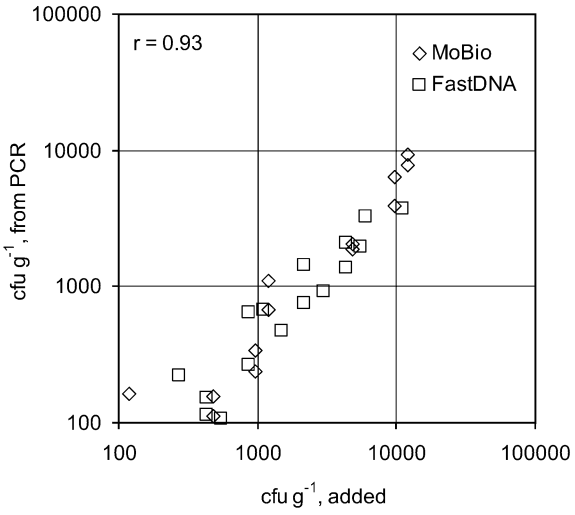

Fig. 2 Real-time PCR results from cardboard samples spiked with vegetative cells. The correlation coefficient (*r*) is for both data sets. $n = 13$ for MoBio; $n = 16$ for FastDNA

was ambiguous (Table 4). Culturing results from shocked and non-shocked suspensions were similar, indicating that cells were present as spores.

Discussion

The aim of this study was to detect *B. cereus* group cells from naturally contaminated cardboard samples by PCR. With DNA isolation kits, only 0.1 g of cardboard could be added, which is a very small sample volume. Therefore, attempts were made to homogenize larger sample volumes and then concentrate them. Samples can be concentrated by centrifugation [24], filtration [9] or using magnetic beads [11, 15]. In our study, both centrifugation and filtering were tested, but filtration proved to be impossible because of filter-clogging. The method based on centrifugation gave very low yields of added bacteria, due to losses of bacteria in both homogenization and centrifugation steps. Apajalahti et al. [5] concentrated bacterial cells from the gastrointestinal tract of chickens for DNA extraction and showed that lower than 30,000 *g* forces caused incomplete pelleting and poor bacterial recovery. The kit-based DNA extraction methods used here gave much higher yields, despite the low sample volume (Fig. 2, Table 3). With the kits, an average yield of 50% of added cells was obtained in spiking experiments; and the results correlated with added cfu values at 100–10,000 cfu g^{-1}. The two kits, MoBio and FastDNA, did not differ significantly in their efficiency. The FastDNA kit has also been shown to be suitable for detecting fungi from corrugating paper by PCR [30].

Since the aim was to detect both vegetative cells and spores, another question was which treatment would be strong enough to release DNA from spores but not so strong that it destroys the DNA released from vegetative

Table 3 Plate count and PCR results of the numbers of *B. cereus* group bacteria in cardboard samples. PCR results given are means for five subsamples analyzed in separate experiments (standard errors of the means in parentheses). Two separate experiments of plate counts were done and results are shown as a range of variation, since API tests could not identify the colonies with certainty

Sample number	Sample description	Mass ($cm^2 g^{-1}$)	*B. cereus* group bacteria (cfu g^{-1})		
			Plate count	PCR, MoBio	PCR, Fast DNA
1	Fluting	61	< 100	< 100	< 100
2	Dry extra pulp	–	< 100	< 100	< 100
3	Paper board	93	< 100	< 100	< 100
4	Fluting, virgin fiber	96	< 100	< 100	< 100
5	Liner, virgin fiber	56	< 100	< 100	< 100
6	Fluting, recycled fiber	74	100–360	780 (200)	1350 (320)
7	Liner, recycled fiber	82	100–300	1720 (810)	4220 (1040)
8	Newsprint	211	< 100–300	160 (20)	240 (50)
9	Fine paper	91	< 100	< 100	< 100

Table 4 API results from verified colonies of three cardboard and paper samples

Sample	Species	Identity (%)	API remark
Fluting, recycled fiber	*B. mycoides*	55	Good identification of the genus
	B. cereus	94	Acceptable identification
	B. mycoides	88	Acceptable identification
	B. mycoides	88	Acceptable identification
	B. mycoides	60	Good identification of the genus
	B. mycoides	78	Good identification of the genus
Liner, recycled fiber	*B. cereus*	94	Good identification
	B. mycoides	42	Excellent identification of the genus
	B. mycoides	54	Good identification of the genus
	B. mycoides	67	Doubtful profile
	B. cereus	93	Very good identification of the genus
	B. cereus	68	Very good identification of the genus
Newsprint	*B. mycoides*	80	Very good identification of the genus
	B. mycoides	80	Very good identification of the genus
	B. mycoides	80	Very good identification of the genus
	B. mycoides	80	Good identification of the genus
	B. mycoides	80	Good identification of the genus
	B. mycoides	80	Very good identification of the genus
	B. mycoides	80	Very good identification of the genus
	B. cereus	64	Very good identification of the genus
	B. mycoides	80	Very good identification of the genus
	B. anthracis	85	Excellent identification of the genus
	B. anthracis	85	Excellent identification of the genus

cells. Releasing DNA from spores can be done by microwave treatment [24, 43], sonication [10], mechanical breaking (cell milling) [28] and germination with subsequent lysis of vegetative cells [28]. In our study, mechanical lysing for 3 min gave the same yield of DNA, measured by real-time PCR, for both vegetative cells and spores in pure cultures. In cardboard-spiking experiments, however, results from adding spores were about five times lower than results from adding the same amounts of vegetative cells. Even activation of spores by heat-treatment or L-alanine did not increase the yield. *B. cereus* group cells are expected to exist in cardboard as spores. Since they were detected from cardboard samples in higher numbers than by culturing, it is speculated that spores which have just been made in the laboratory could be in a different physiological state than those in cardboard samples and thus more difficult to lyse. The spore core of dormant *B. cereus* and *B. subtilis* can be in a glassy state [31]. The physiological state of spores in cardboard and paper and the failure to detect added spores from spiked samples requires further study to be clarified.

Three out of nine unspiked cardboard and paper samples were shown to be naturally contaminated with *B. cereus*, based on both culturing and real-time PCR (Table 3). For pure cultures, very sensitive real-time PCR assays with a wide dynamic range have been established, but the results from different sample matrices are variable. Even though PCR and even real-time PCR methods are widely used, many studies are done with artificially contaminated sample materials and studies testing real contaminated samples are rare. Hein et al. [23] contaminated three different cheese types artificially with *Staphylococcus aureus* and the detection level was, depending on the cheese matrix, 10–175 organisms g^{-1} by detecting the *nuc* gene in a

LightCycler system. Lower numbers did not give consistent results. The coefficients of correlation between cfu and *nuc* gene copy numbers ranged from 0.979 to 0.998. The detection level of *Listeria monocytogenes* from spiked milk samples was approximately 200 cfu ml^{-1} when DNA was isolated with bacterium-binding beads and cell standard curves were reproducible and linear [33]. *Salmonella enterica* has been detected from naturally contaminated poultry samples by real-time PCR, but the samples were pre-enriched before DNA extraction, thus preventing quantification [16]. In our study, the quantitative detection limit of PCR was set to crossing point 31, which corresponded theoretically to 100 cfu g^{-1}. Because the average yield in spiking experiments was 50%, a detection level of approximately 200 cfu g^{-1} was obtained in practice (Fig. 2). Nevertheless, even lower numbers of added bacteria repeatedly produced a specific amplification product. Thus the detection level was lower, but reliable quantification of cells was not possible under 100–200 cfu g^{-1}.

The theoretical detection limit of culturing was 100 cfu g^{-1} paper board in this study, since the dilution made in homogenization was 10^{-2}. Quantifying *B. cereus* by culturing combined with API tests gave uncertain results, since not all strains from paper and cardboard samples could be identified by the API identification patterns (Table 4). API patterns are mainly based on clinical strains; and it has been shown that they cannot identify environmental strains reliably [40]. Suihko and Stackebrandt [40] also showed that strains originating from the pulp and paper industry often cannot be identified by ribotyping. Sequencing all isolates would be more reliable, but it is not practical as a routine measure.

The numbers of *B. cereus* group cells in the samples detected by PCR were between 10^2 g^{-1} and 10^3 g^{-1} (Table 3). More exact values are difficult to obtain. In PCR, quantification of *B. cereus* is complicated by the fact that different *B. cereus* strains have been shown to have different copy numbers (9–12) of rRNA operons [27]. *B. thuringiensis* subsp. *canadensis* was shown to have eight rRNA gene operons [13]. Based on our measurements made with different *Bacillus* species, it seems that copy numbers do, indeed, vary (Fig. 1A). If copy numbers vary between eight and 12, the maximum difference in the results would be 2.25-fold. In addition, a variation in results from both PCR and culturing is caused by the uneven distribution of bacteria in paper and board [41]. To obtain representative samples, large samples were cut into pieces and mixed before taking subsamples, but it is inevitable that different subsamples had different bacterial numbers.

The fact that PCR gave higher results than culturing could also be due to DNA from dead or non-cultivable cells giving a signal. DNA analyses are often questioned because of their ability to detect non-viable cells, but it has been shown, at least with *Vibrio vulnificus* and *Campylobacter jejuni*, that even non-cultivable cells can still be infectious [12, 29]. Furthermore, the presence of dead cells indicates contamination of the samples.

In conclusion, real-time PCR results from cardboard samples were reproducible and in concordance with culturing. It is concluded that this method can be reliably used in rapid screening for the presence of *B. cereus* group cells in samples and for giving an order of magnitude of the numbers of bacteria. If necessary, positive samples may then be studied in more detail. There are uncertainties in the absolute quantification of *B. cereus* group bacteria from cardboard samples: namely the variation in 16S rDNA copy numbers and the failure to detect spiked spores to the same extent as vegetative cells. Nevertheless, the culturing method has equal uncertainties, as discussed above. The clear advantage of PCR over culturing is that results can be obtained in 1 day instead of several days, as required for the culturing and phenotypic verification of isolates.

Acknowledgements We are grateful to Päivi Lepistö for excellent laboratory work and to the paper mills for providing the samples. This work was partly supported the by National Technology Agency of Finland (TEKES) and the Finnish food and paper industries.

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