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Diagnostic Real-Time PCR Assays for the Detection of Emetic *Bacillus cereus* Strains in Foods and Recent Food-Borne Outbreaks▽†

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Cereulide-producing *Bacillus cereus* can cause an emetic type of food-borne disease that mimics the symptoms provoked by *Staphylococcus aureus*. Based on the recently discovered genetic background for cereulide formation, a novel 5′ nuclease (TaqMan) real-time PCR assay was developed to provide a rapid and sensitive method for the specific detection of emetic *B. cereus* in food. The TaqMan assay includes an internal amplification control and primers and a probe designed to target a highly specific part of the cereulide synthetase genes. Additionally, a specific SYBR green I assay was developed and extended to create a duplex SYBR green I assay for the one-step identification and discrimination of the two emesis-causing food pathogens *B. cereus* and *S. aureus*. The inclusivity and exclusivity of the assay were assessed using a panel of 100 strains, including 23 emetic *B. cereus* and 14 *S. aureus* strains. Different methods for DNA isolation from artificially contaminated foods were evaluated, and established real-time assays were used to analyze two recent emetic food poisonings in southern Germany. One of the food-borne outbreaks included 17 children visiting a day care center who vomited after consuming a reheated rice dish, collapsed, and were hospitalized; the other case concerned a single food-poisoning incident occurring after consumption of cauliflower. Within 2 h, the etiological agent of these food poisonings was identified as emetic *B. cereus* by using the real-time PCR assay.

Bacillus cereus is increasingly recognized as the etiological agent of gastrointestinal and nongastrointestinal diseases. Two clinical pictures connected to food poisoning, diarrhea and emesis, can be distinguished. Heat-labile enterotoxins elicit diarrhea, while a heat-stable depsipeptide toxin, called cereulide, provokes emesis (for a review, see the works of Granum [18], Ehling-Schulz et al. [13], and Schoeni and Wong [43]). In general, both types of food-borne disease are relatively mild and self-limiting. Nevertheless, during the last few years, severe forms of disease caused by emetic *B. cereus* have occasionally involved hospitalization or even death (11, 27). Due to the increasing number of reports of food-borne disease, especially of severe cases, fast detection methods are required for diagnostic purposes as well as for the prevention of food contamination and food-borne outbreaks.

The true incidence of *B. cereus* food poisoning is unknown for a number of reasons, including misdiagnosis of the disease, which is symptomatically similar to other types of food poisoning. For example, the symptoms caused by emetic *B. cereus* resemble those caused by *Staphylococcus aureus* (13). Standard detection methods with enrichment, plating, and further identification require 3 days for *B. cereus* and up to 6 days for *S. aureus* (4, 30, 37). For the conclusive identification of the emetic toxin cereulide from *B. cereus* strains, additional laborious methods, such as high-performance liquid chromatography connected to ion trap mass spectrometry, are necessary (19). Only recently has progress been made in the development of molecular detection systems for emetic *B. cereus* strains. The first molecular assays, based on conventional PCR systems, for the identification of emetic strains and the detection of the toxin gene have been described previously (e.g., by Ehling-Schulz et al. [14, 15]). However, a major drawback of conventional PCR is the requirement for post-PCR analysis by gel electrophoresis, which is time consuming and bears the risk of false-positive results due to laboratory contamination.

The introduction of real-time PCR provides the opportunity for the rapid detection of pathogens in food and clinical settings. Apart from saving time, real-time PCR is highly specific and sensitive and offers the potential for quantification (25). The risk of cross-contamination is significantly reduced, and high-throughput performance and automation are possible since no post-PCR manipulations are required.

Recently, the biosynthetic code for nonribosomal synthesis of the emetic toxin cereulide has been deciphered (16, 26). We used this genetic information to develop a TaqMan-based, real-time PCR assay. The assay targets a highly specific part of the cereulide synthetase (*ces*) genes and includes an internal amplification control (IAC). In addition, a duplex SYBR green I real-time PCR assay for one-step differentiation between emetic *B. cereus* and *S. aureus* is reported. These novel diagnostic assays were successfully applied to identify of the causative agent of recent emetic food-poisoning outbreaks.

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TABLE 1. Bacterial species used to test the inclusivity and exclusivity[a] of the SYBR green I and TaqMan real-time PCR assays

Bacterial species (no. of species)	No. of strains tested	Result with the indicated detection method		
		TaqMan PCR	SYBR green simplex	SYBR green duplex
Bacillus cereus group (n = 5)				
Non-emetic *Bacillus cereus*	16	−	−	−
Emetic *Bacillus cereus*	23	+	+	+
Bacillus thuringiensis	4	−	−	−
Bacillus mycoides	4	−	−	−
Bacillus weihenstephanensis	4	−	−	−
Bacillus anthracis	4	−	−	−
Bacillus sp. (n = 5)				
Bacillus brevis	3	−	−	−
Bacillus subtilis	1	−	−	−
Bacillus licheniformis	3	−	−	−
Bacillus amyloliquefaciens	1	−	−	−
Bacillus megaterium	1	−	−	−
Non-*Bacillus* species (n = 7)				
Staphylococcus aureus	14	−	−	+
Clostridium perfringens	3	−	−	−
Listeria monocytogenes	4	−	−	−
Campylobacter sp.	3	−	−	−
Escherichia coli (including serovar O157)	4	−	−	−
Salmonella sp.	4	−	−	−
Yersinia enterocolitica	4	−	−	−
Total no. of strains	100			

[a] Inclusivity and exclusivity are defined according to Malorny et al. (29).

MATERIALS AND METHODS

Bacterial strains. The reference strain for emetic toxin, *B. cereus* F4810/72, was used to develop both real-time PCR systems; in addition, *S. aureus* strain WS2608 was included to establish the duplex real-time PCR assay. Bacterial strains (n = 100) used to assess the inclusivity and exclusivity of the real-time PCR assays are listed in Table 1. Details on the origin of the bacterial strains included in this study are provided in the supplemental material. Strains were grown either on Luria-Bertani (LB) agar or in LB broth (10 g tryptone, 5 g NaCl, 5 g yeast extract, 15 g agar per liter) as described previously (14).

DNA isolation. Total DNA from strains used to test the specificity of the developed PCR assays was isolated from overnight cultures using the AquaPure genomic DNA isolation kit (Bio-Rad, Germany) according to the manufacturer's instructions. For the determination of the detection limit of the real-time PCR assays, *B. cereus* DNA from the emetic reference strain F4810/72 and the *S. aureus* strain WS2608 were isolated by phenol-chloroform extraction as described previously (9, 14) to obtain pure high-molecular-weight genomic DNA. For sensitivity and specificity tests, total DNA from food samples was isolated using either the AquaPure genomic DNA isolation kit (Bio-Rad, Germany) or the NucleoSpin food kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. In addition, DNA from food samples was extracted using a simple boiling method. In brief, cells from 1 ml enriched samples were harvested by centrifugation and cell pellets were resuspended in 300 μl sterile Milli-Q, boiled for 10 min, and then cooled on ice. After cell and food residues were pelleted, a 5-μl aliquot of the supernatant was used as PCR template.

Primer and TaqMan probe design. Primers and probes used in this study are listed in Table 2. The emetic *B. cereus*-specific TaqMan primers and probe as well as the SYBR green I primers were derived from the cereulide synthetase (*ces*) gene sequence (accession no. DQ360825), thereby targeting a region of the *ces* genes shown to be highly specific for emetic *B. cereus* (12). TaqMan and IAC primers and probes were designed using Primer3 software (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For the amplification of an *S. aureus*-specific genomic DNA fragment, primers targeting the *tuf* gene (31), which encodes the elongation factor Tu, were included in the SYBR green I-based real-time PCR assay. The specificity of the sequences was tested by searches against NCBI's nonredundant database using the BLASTN algorithm (5). All primers and probes were purchased from MWG Biotech (Germany). The target probe for emetic *B. cereus* was labeled at the 5′ end with the reporter dye 6-carboxyfluorescein, and the IAC probe was labeled at the 5′ end with 5-hexachloro-6-carboxyfluorescein. Both probes were labeled at the 3′ ends with tetramethyl-6-carboxyrhodamine.

Internal amplification control. The commercially available plasmid pUC19 (Fermentas, Germany) was used as the IAC without any modifications. The TaqMan primers and probe were placed in the pMB1 replicon *rep* (Table 2). The optimal IAC concentration was assessed to be approximately 170 copies per PCR.

Real-time PCR assays. A typical 25-μl PCR mixture for the TaqMan-based PCR assay contained 12.5 μl Brilliant QPCR Multiplex Mastermix (Stratagene), 0.5 μM of each primer (for emetic *B. cereus* and IAC each), 0.2 μM emetic *B. cereus* probe (ces_TaqMan_probe) and 0.2 μM IAC probe (IAC_probe), approximately 170 copies of plasmid DNA pUC19 (Fermentas, Germany), and 5 μl of the sample DNA. No-template controls that contained 5 μl Tris-EDTA buffer instead of DNA were included in each run to detect any contamination. Typical cycling conditions under a Stratagene MX3000P real-time PCR system (Stratagene) were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 55°C for 60 s. The maximum ramp rate of the Stratagene MX3000P real-time PCR system was 2.5°C/s. A typical 25-μl PCR mixture for the SYBR green I real-time PCR assay consisted of 12.5 μl SYBR Premix Ex *Taq* (Takara Bio, Inc., Japan), 1 to 5 μl of template DNA (depending on extraction method), 0.3 μM for each *B. cereus* primer (ces_SYBR_F and ces_SYBR_R), and 0.12 μM for each *S. aureus* primer (sa_SYBR_F and sa_SYBR_R). Typical cycling conditions using a Stratagene MX3000P real-time PCR system (Stratagene) were 95°C for 10 s, followed by 45 cycles at 95°C for 10 s and 60°C for 30 s. The same conditions were used with the SmartCyclerII system (Cepheid), but with the ramp rate set on maximum (10°C/s) for the simplex detection of emetic *B. cereus* strains and altered to 3°C/s for simplex and duplex detection involving the primers specific for *S. aureus*. In the subsequent melting curve analysis, the temperature was raised from 60°C to 95°C with a ramp rate of 0.2°C/s. The specificity and robustness of

TABLE 2. Primers and probes used in this study

Primer	Sequence from 5′ to 3′ end[a]	Position	Sequence reference	Amplicon length	Reference
ces_SYBR_F	CACGCCGAAAGTGATTATACCAA	8743–8765	DQ360825	176 bp	This study
ces_SYBR_R	CACGATAAAACCACTGAGATAGTG	8895–8918			
sa_SYBR_F	CGTGTTGAACGTGGTCAAATCA	389–410	AF298796	262 bp	Derived from reference 30
sa_SYBR_R	CACCTTCGTCTTTTGATAATACG	628–650			
ces_TaqMan_for	CGCCGAAAGTGATTATACCAA	8745–8765	DQ360825	103 bp	This study
ces_TaqMan_rev	TATGCCCCGTTCTCAAACTG	8828–8847			
ces_TaqMan_probe	FAM-GGGAAAATAACGAGAAATGCA-TAMRA	8798–8818			
IAC_for	GCAGCCACTGGTAACAGGAT	1216–1235	L09137	118 bp	This study
IAC_rev	GCAGAGCGCAGATACCAAAT	1314–1333			
IAC_probe	HEX-AGAGCGAGGTATGTAGGCGG-TAMRA	1240–1259			

[a] FAM, 6-carboxyfluorescein; HEX, 5-hexachloro-6-carboxyfluorescein; TAMRA, tetramethyl-6-carboxyrhodamine.

the assays were tested in two independent labs on two different cycler systems (Stratagene MX3000P and SmartCyclerII), revealing consistent results and similar detection limits.

Standard curves and efficiency. Total DNA from the strain F4810/72 used for the standard curves was isolated with phenol-chloroform extraction as described above. The concentration of DNA was determined by using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies). Standard curves were generated using a tenfold dilution series of DNA ranging from 600 ng to 6 fg for the TaqMan assay on the Stratagene MX3000P as well as for the SYBR green I simplex assay on the SmartCyclerII. Cycle threshold (C_T) values were calculated with the standard algorithms provided with the two cycler systems. The PCR efficiency (E) was calculated according to the method of Pfaffl (34) from the standard curve with the equation $E = 10^{-1/\text{slope}} - 1$. Additionally, an assay precision test according to the method of Malorny et al. (29) was performed for the TaqMan and the SYBR green I simplex real-time assays.

Standard reference culture methods. Cell counts in food samples were determined in accordance with standard reference culture methods recommended by food authorities (§64 Lebensmittel-, Bedarfsgegenstände- und Futtermittelgesetzbuch [LFGB], International Organization for Standardization [ISO], and the U.S. Food and Drug Administration [FDA]). In brief, 25 g of food sample was homogenized in a stomacher (Lab-Blender 400; Kleinfeld Labortechnik, Germany) with 225 ml BHIG broth (brain heart broth [Merck, Germany] supplemented with 0.1% glucose) and was incubated at 37°C for 24 h without shaking. Serial dilutions of the enrichment were plated on LB, PEMB (polymyxin-egg yolk-mannitol-bromothymol blue agar [Oxoid, Germany]), and Baird Parker agar and incubated at 37°C as prescribed.

Artificial inoculation of food samples. Rice and pasta were purchased from local food stores in Germany. Food samples were cooked and tested for the absence of any naturally occurring contamination with *B. cereus* and *S. aureus* by the standard reference culture methods described above. As no *B. cereus* and *S aureus* bacteria were detected, the food was used for artificial contamination. Twenty-five grams of cooked rice or pasta was homogenized with 225 ml BHIG broth, and the mixture was inoculated with serial dilutions of F4810/72 and/or WS2608 overnight culture in the range of 10^0 to 10^3 CFU/g food. Enrichment was carried out at 37°C without shaking. Samples were taken after 0, 2, 4, and 6 h of enrichment for DNA isolation and the determination of cell counts on LB, PEMB, or Baird Parker agar. One-milliliter aliquots of enriched samples were centrifuged (13,000 × *g* for 4 min), and pellets were stored at −20°C until DNA isolation and PCR analysis. DNA isolation was carried out using the (i) AquaPure genomic DNA isolation kit (Bio-Rad, Germany), (ii) NucleoSpin food kit (Macherey-Nagel, Germany), and (iii) simple boiling method in parallel.

Investigation of two emetic outbreaks. DNA for real-time PCR was isolated directly from the food remnants connected to two recent emetic outbreaks in southern Germany by using the commercial DNA isolation kits and the simple boiling method as described above. Additional cell counts were determined with the standard reference culture methods but without enrichment. Cereulide was extracted from the food remnants as follows: 5 g sample was homogenized in 5 ml sterile Milli-Q and was autoclaved (20 min at 21°C). Ten microliters of the supernatant was then tested in a HEp-2 cell culture assay as described previously (16). The commercially available enzyme-linked immunosorbent assay system VIDAS Staph enterotoxin II (bioMérieux, France) was used to test the food remnants for *S. aureus* enterotoxins according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Design and analytical accuracy of real-time PCR assays for emetic *B. cereus*. Diagnosis of *B. cereus* linked to the emetic type of food poisoning was hampered by the lack of genetic information on the toxin genes. Only recently were the genes encoding the enzymatic machinery required for biosynthesis of the emetic toxin cereulide identified and characterized (12, 16). Based on this information, primers and a probe were designed for the detection of emetic *B. cereus* in food by real-time PCR (Table 2). In principle, two different chemistries are available for real-time detection of PCR products: fluorescent probes that bind specifically to certain DNA sequences and fluorescent dyes that intercalate in any double-stranded DNA. Within this study, a 5′ nuclease (TaqMan) real-time PCR for diagnostic purposes as well as a SYBR green I-based system, representing an interesting economic alternative for high-throughput screening purposes, was developed. The primers of both PCR systems and the TaqMan probe anneal within an unusual insertion in the *cesA1* domain which encodes a novel type of α-ketoreductase that performs chiral reduction of α-ketoacyl-S-carrier proteins as opposed to the typical ketoreductases found in polyketide synthetases (26). This insertion has been shown to be highly specific for emetic *B. cereus* by hybridization studies and database analysis (12). The primers used for the SYBR green I detection of these strains were selected in such a way as to give optimal results when combined with the primers specific for the *S. aureus* detection in the duplex assay, whereas the primers and the probe used for the TaqMan assay were designed with the Primer3 software and are located within the sequence amplified by the primers used for the SYBR green I assay. As the use of IAC in diagnostic PCR is becoming mandatory (20, 28), an IAC was included in the TaqMan assay (for details, see Materials and Methods). Such an internal control indicates the presence of PCR amplification inhibitors or the malfunction of the thermal cycler (3, 42).

The inclusivity and exclusivity (according to Malorny et al. [30]) of the TaqMan and SYBR green I assays were assessed using a test panel of 100 strains, including 23 emetic strains from different origins (Table 1; see Table S1 in the supplemental material). Only the emetic *B. cereus* strains showed positive results in the above-mentioned assays; all of the other strains tested negative. No cross-reaction was observed for any of the strains included in the test panel (Table 1); nevertheless the strain set included nonemetic *B. cereus* strains that carry potential *nrps* genes and known NRPS-carrying bacteria like the gramicidin and tyrocidine producer *Bacillus brevis*. The detection limits of the developed TaqMan (including the IAC) and the SYBR green I assays were determined in two independent runs with tenfold serial dilutions from purified genomic DNA of the reference strain for emetic toxin F4810/72. The limits were 0.6 pg for the TaqMan assay and 0.06 pg for the SYBR green I assay. The PCR amplification efficiency, calculated according to the method of Pfaffl (34), was 108% for the TaqMan assay and 91.5% for the SYBR green I assay (data not shown). Results for the detection probability from dilution series from pure culture are provided in the supplemental material. In addition, serial dilutions of *B. cereus* DNA were used to determine the precision of both assays as described previously by Malorny et al. (29). The repeatability standard deviation s_r was calculated by using the measured mean C_T values of 10 replicates from four consecutive runs (Table 3). The calculated s_r values for the TaqMan (1.5 to 2.2%) and the SYBR green I assay (1.5 to 3.3%) indicate the high precision of the assay.

Recently, it has been shown that the cereulide synthetase gene cluster is located on a 208-kb megaplasmid that has high homology to the plasmids pXO1 of *Bacillus anthracis* and pBc10987 of *B. cereus* ATCC 10987 (12). According to Rasko et al. (35, 36), these are low-copy plasmids with copy numbers of between 1 and 3 per cell. Assuming similar numbers for the pBCE4810 plasmid, the detection limit of the real-time PCR assay (for pure cultures) would correspond to approximately 10 genomic equivalents for the SYBR green I simplex PCR assay and 100 genomic equivalents for the TaqMan assay (de-

TABLE 3. Mean C_T and s_r of tenfold serially diluted DNA from the reference strain for emetic toxin F4810/72 in the TaqMan real-time PCR assay in the presence of 170 IAC copy numbers and in the SYBR green simplex PCR assay

Amt of emetic *B. cereus* DNA/PCR	Mean C_T and s_r using[a]:		
	SYBR green simplex	TaqMan PCR	
		ces-specific probe	IAC-specific probe
600 ng	14.82 ± 0.24	18.98 ± 0.37	34.03 ± 2.76
60 ng	18.38 ± 0.27	22.60 ± 0.40	31.14 ± 2.43
6 ng	20.72 ± 0.46	25.73 ± 0.54	30.49 ± 1.48
600 pg	23.61 ± 0.74	28.93 ± 0.59	30.80 ± 1.18
60 pg	29.17 ± 0.52	32.20 ± 0.49	30.87 ± 1.13
6 pg	32.64 ± 1.07	35.41 ± 0.63	31.07 ± 1.41
0.6 pg[b]	35.45 ± 0.54	37.69 ± 0.83	30.96 ± 1.26

[a] Results are shown as $C_T \pm s_r$. C_T values were calculated with the standard algorithms provided with the two cycler systems; s_r values were calculated from 10 replicates from four consecutive runs.
[b] Approximately 100 genomic equivalents correspond to 0.6 pg.

duced from the genome size of *B. cereus* ATCC 10987 and calculated with the formula proposed by Rodríguez-Lázaro et al. [39]).

Duplex real-time PCR assay for one-step differentiation of two emesis-causing pathogens: *B. cereus* and *S. aureus*. *B. cereus* and *S. aureus* are broadly distributed microorganisms that are often transferred to foodstuff. Typical cell counts in food samples connected to food poisoning are 10^5 to 10^8 CFU *B. cereus*/g (18) or 10^6 to 10^8 CFU *S. aureus*/g (21), sometimes less. Microbial norms in most countries tolerate low numbers of these pathogens, but threshold values often depend on the foodstuff in question, its further preparation (reheating or ready to eat), and the potential consumer (young, old, or immunocompromised people). For example, threshold values for *B. cereus* in Germany vary from 10^2 CFU/g in baby food to 10^4 CFU/g in spices (6) and those for *S. aureus* in France vary from 0 CFU/g in semicanned food to 10^3 CFU/g in some raw milk cheeses (24). Since the emetic syndrome caused by *B. cereus* cannot be differentiated symptomatically from intoxications with *S. aureus* (13), one-step detection and differentiation between these two pathogens would improve the speed and accuracy of diagnosis significantly. Of food-poisoning cases in which *S. aureus* had been suspected to be the causative agent, in only 2% (1 out of 50) was *S. aureus* identified (D. Mäde [Halle, Germany], personal communication). However, *B. cereus* was the main cause of food-borne disease from mass-catered food prepared by and served to the German Armed Forces from 1985 to 2000 and the most common pathogen isolated from food-borne illness in 1990 in Norway (2, 23). It is therefore tempting to speculate that a significant portion of those cases in which the causative agent remained unknown were actually elicited by emetic *B. cereus*.

SYBR green I-based duplex PCR has been shown to be suitable for the one-step detection and differentiation of food and plant pathogens (1, 8, 22). Product accumulation during PCR is monitored by the double-stranded DNA-specific cyanine dye SYBR green I, followed by the identification of amplicons by melting curve analyses. The melting temperature (T_m) of an amplicon depends on its GC content, length, and sequence characteristics, whereby different PCR products can be distinguished (38). We used this discriminatory feature to design a duplex real-time PCR assay for the one-step differentiation between emetic *B. cereus* and *S. aureus*.

The emetic *B. cereus*-specific primers (ces_SYBR_F and ces_SYBR_R) designed for the simplex assay were used in combination with *S. aureus*-specific primers (sa_SYBR_F and sa_SYBR_R) derived from published primers targeting the *tuf* gene (31). These primers allowed the amplification of PCR products with distinct melting temperature values, resulting in the formation of two distinct peaks representing the two targets (Fig. 1A). The 176-bp amplicon of emetic *B. cereus* (T_m, 80.0°C; GC content of 35%) could be clearly separated from the 262-bp amplicon of *S. aureus* (T_m, 83.5°C; GC content of 40%). Melting points showed a higher degree of variation in the duplex real-time PCR assay than in the simplex assay, but both amplicons were still clearly distinguishable from each other (Fig. 1A). The T_m in duplex PCR was 79.3 ± 0.2°C for the emetic *B. cereus*-specific amplicon and 83.4 ± 0.2°C for the *S. aureus*-specific amplicon. Detection limits in the SYBR green reactions were 0.06 pg for emetic *B. cereus* and 5 pg for *S. aureus*. The robustness of the duplex real-time PCR was tested with serial tenfold dilutions of DNA from one pathogen in the presence of constant amounts of DNA from the other pathogen. The results of two independent runs were compa-

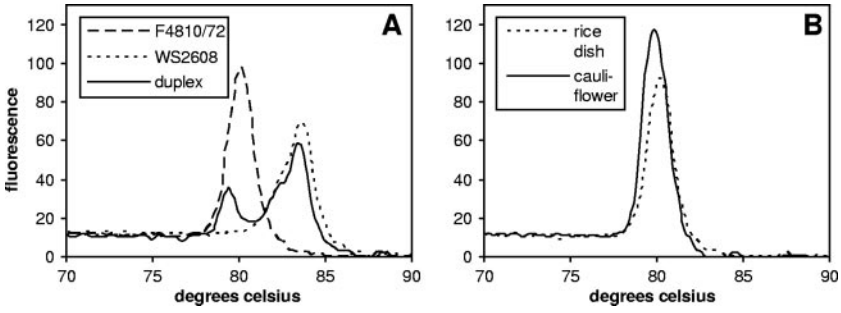

FIG. 1. (A) Melting curves of SYBR green I real-time PCR products used for the detection of emetic *Bacillus cereus* (F4810/72) and *Staphylococcus aureus* (WS2608). DNA was isolated with the NucleoSpin food kit from a duplex enrichment experiment with artificial inoculated rice after 6 h. Cell counts correspond to 10^8 CFU/g F4810/72 and 10^7 CFU/g WS2608 (Table 5). The melting point of emetic *B. cereus* in the simplex reaction is about 80.0°C and shifts down to 79.3°C in the duplex reaction, whereas the melting point for *S. aureus* is about 83.4°C in both reactions. (B) Melting curves of the duplex SYBR green I real-time PCR products from the food-borne outbreaks (for details on the outbreaks, see Results and Discussion). Only the amplicon specific for emetic *B. cereus* could be detected in both cases.

TABLE 4. Robustness of the duplex SYBR green I real-time PCR for emetic *B. cereus* and *S. aureus*

Amt of DNA used as template		Species detected[a]	
Emetic *B. cereus*	*S. aureus*	Emetic *B. cereus*	*S. aureus*
50 ng	0.5 pg	+	−
50 ng	50 pg	+	+
50 ng	50 ng	+	+
50 pg	50 ng	+	+
0.5 pg	50 ng	+	+

[a] +, species was detected; −, species was not detected.

rable and are shown in Table 4. In brief, in the presence of 50 ng of *S. aureus* DNA, 0.5 pg of emetic *B. cereus* DNA could be detected, while amounts as low as 5 pg of *S. aureus* DNA could be detected in the presence of 50 ng of emetic *B. cereus* DNA.

Real-time analysis of artificially contaminated foods. Various methods have been described in the literature for DNA extraction from foods for real-time PCR, since it is highly sensitive towards inhibitory substances originating from the food matrix (32). Often these methods include a complexing step (e.g., cetyltrimethylammonium bromide), magnetic beads, or commercially available kits (33, 40, 41), but also simple methods like boiling have been reported to be suitable (10). In this study, two commercially available kits (see Materials and Methods) and a simple boiling method were used for DNA isolation from artificially contaminated foods to evaluate the robustness of the real-time assays.

Different foods were tested for the absence of natural contamination with *B. cereus* and were then used for artificial contamination experiments. Cooked rice and pasta, which had been inoculated with different amounts of the emetic toxin reference strain F4810/72, were analyzed by real-time PCR and in parallel by standard reference culture methods at different enrichment time points (for details, see Materials and Methods). The experiment was repeated three times independently, and DNA was isolated from two of these experiments. Without enrichment, the detection limit of the TaqMan-based assay including the IAC was 10^5 CFU/g (17 CFU per reaction) artificially contaminated rice for the boiling method and 10^3 CFU/g (2 CFU per reaction) for the kit-based DNA isolation method. With SYBR green I chemistry, the detection limit was 10^3 CFU/g rice for the boiling method and 10^1 CFU/g with the kit-based DNA isolation method. Considering the increase of cell numbers during enrichment (Table 5), a reliable detection of 10^0 CFU of emetic *B. cereus* per gram of food was possible with the simple boiling method after 6 h of enrichment for the TaqMan assay and after 4 h of enrichment for the SYBR green I assay. Using the kit-based DNA isolation methods, the enrichment time could be reduced to 2 h for the SYBR green I assay and to 4 h for the TaqMan assay. But as the kit-based DNA isolation method requires about 2 h (including incubation time), a longer enrichment time in combination with the simple boiling method can accelerate the results of the real-time PCR and lower the cost. Spiking experiments performed with artificially contaminated pasta revealed detection limits similar to those depicted for the rice experiments (data not shown).

In addition, artificial inoculation experiments were conducted to determine the detection limit of the duplex real-time PCR assay. Rice was inoculated with the emetic reference strain F4810/72 or the *S. aureus* strain WS2608 or both strains together. Cell counts are provided in Table 5. DNA from these enrichment experiments was isolated with the NucleoSpin food kit. After 6 h of enrichment both pathogens could be detected simultaneously from the same artificially contaminated rice by the SYBR green I duplex assay.

In summary, the diagnostic accuracy of the real-time assays was determined and showed that the developed assays are suitable for the detection of 10^0 CFU/g emetic *B. cereus* in foods after a short enrichment time (4 to 6 h), while higher cell numbers (10^1 to 10^3 CFU/g) can be detected directly from food samples without the need for further enrichment steps. Since the typical cell counts reported in foods incriminated in emetic outbreaks have been reported to be about 10^5 to 10^8 CFU *B. cereus*/g (18), samples from emetic food poisonings can be processed and analyzed within 1.5 to 4 h, depending on the DNA isolation method applied. In food samples connected to *S. aureus* intoxications, cell counts of between 10^5 and 10^6 CFU/g have been reported (7). The SYBR green duplex assay allowed the detection of 10^3 CFU *S. aureus*/g food after 4 h of enrichment and after 6 h of enrichment when emetic *B. cereus* was present in the same enrichment. However, due to the heat stability of the emesis-causing toxins from both pathogens, molecular analysis by real-time PCR should be supplemented

TABLE 5. Cell counts of the enrichment experiment with the reference strain for emetic toxin *B. cereus* F4810/72 and *S. aureus* WS2608 in rice[a]

Enrichment duration	Cell count at inoculation level (CFU/g) for:							
	Enrichment with fresh overnight culture of F4810/72				Duplex enrichment with fresh overnight culture of F4810/72 and WS2608			
	10^0	10^1	10^2	10^3	10^3 (only F4810/72)	10^3 (only WS2608)	10^3 (F4810/72 and WS2608 together)	
					B. cereus	*S. aureus*	*B. cereus*	*S. aureus*
0 h	–[b]	2.0×10^1	3.3×10^2	1.8×10^3	2.6×10^3	7.5×10^3	2.5×10^3	7.9×10^3
2 h	6.0×10^1	1.3×10^2	*1.8 × 10³*	*1.6 × 10⁴*	*2.0 × 10⁴*	2.1×10^4	*2.8 × 10⁴*	2.2×10^4
4 h	*9.5 × 10²*	*6.6 × 10³*	***1.7 × 10⁵***	***2.2 × 10⁶***	*3.3 × 10⁶*	*4.2 × 10⁵*	*5.2 × 10⁶*	4.1×10^5
6 h	***1.3 × 10⁵***	***6.6 × 10⁵***	***2.5 × 10⁷***	***1.2 × 10⁸***	*2.2 × 10⁸*	*1.5 × 10⁷*	*2.7 × 10⁸*	*1.2 × 10⁷*

[a] Italic numbers indicate a positive result in the SYBR green assay, and bold numbers indicate a positive result in the TaqMan assay. DNA was isolated with the simple boiling method for the simplex detection and with the NucleoSpin food kit for duplex detection.
[b] No colonies were detectable.

by toxin analysis methods, especially in the cases of reheated foods.

Identification of the etiological agent in recent food-borne outbreaks by real-time PCR. In June 2006, 17 children (aged 3 to 5 years) visiting a day care center became sick after eating a rice dish with vegetables. One hour after the meal, the children began vomiting, collapsed, and were hospitalized. Food remnants contained 10^4 CFU *B. cereus*/g rice dish. Emetic *B. cereus* was detected in the rice dish by TaqMan real-time PCR, while *S. aureus* enterotoxin as a causative agent was excluded by using the commercial enzyme-linked immunosorbent assay system VIDAS Staph enterotoxin II (bioMérieux, France). These results were confirmed by the duplex SYBR green I real-time PCR assay developed in this study (Fig. 1B).

The second emetic food poisoning involved one student who consumed cooked cauliflower stored at room temperature for 1.5 days. The student began vomiting 75 min after consumption of about 20 g of the reheated food. DNA was isolated directly from the food remnants and used as template in the TaqMan and the duplex SYBR green I assays. Both assays were positive for emetic *B. cereus*, but the duplex assay was negative for *S. aureus* (Fig. 1B). This is in accordance with the results from conventional enrichment and plating performed in parallel using PEMB agar for selective detection of *B. cereus* and Baird Parker agar for the detection of *S. aureus* (data not shown) as recommended by food authorities (§64 LFGB, ISO, and FDA).

In both food-poisoning cases, the presence of the *B. cereus* emetic toxin cereulide in the food remnants was confirmed by the HEp-2 cytotoxicity assay (16, 17).

Conclusion. We have introduced a TaqMan assay and a SYBR green I real-time PCR assay for the fast and conclusive identification of emetic *B. cereus*. The TaqMan assay includes an IAC to avoid false-negative results and therefore allows its implementation in routine food diagnostic laboratories. The SYBR green I-based assay represents an economically interesting alternative for the analysis of a large number of samples (e.g., in epidemiological studies) and can be extended to a duplex real-time PCR for the one-step differentiation of emetic *B. cereus* and *S. aureus*. The novel real-time PCR assays were shown to be fast, sensitive, and reliable diagnostic tools that complement the existing toxin analysis methods. The developed assays could contribute substantially to determining the true incidence of the emetic type of food poisoning caused by *B. cereus*, especially by reducing the rate of misdiagnosis.

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