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Note

Compact Dry[R] X-BC for the Enumeration of *Bacillus cereus* in Food Samples

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We evaluated the effectiveness of using Compact Dry[R] X-BC (CD-XBC), a ready-to-use and self-diffusing dry medium sheet culture system based on a novel detection principle, for the detection and enumeration of *Bacillus cereus*. All 13 *B. cereus* strains, which were studied for the inclusivity study, grew as blue/green colonies on the CD-XBC. When 3 yeast strains and 103 bacterial strains other than *B. cereus* were tested for the exclusivity study, 5 strains formed white colonies, and 4 strains formed blue/green colonies, while 94 other strains failed to grow. The 4 strains that formed blue/green colonies were *B. thuringiensis*, which is known to have the same biochemical features as *B. cereus*. The CD-XBC method was compared with the MYP agar method (MYP) and the NGKG agar method (NGKG) in 130 artificially contaminated food samples. The correlation coefficients between CD-XBC and MYP, and CD-XBC and NGKG were 0.972 and 0.971, respectively.

Key words : *Bacillus cereus* / Compact Dry[R] X-BC / Chromogenic medium / Ready-to-use medium.

Bacillus cereus is known as a gram-positive spore-forming bacteria that is found widely in nature in the soil and water environment (Ueda et al., 1985). Hence, there are many opportunities for *B. cereus* to contaminate food or the food processing environment (Schoeni and Wong, 2005; Ueda and Kuwabara, 1988; Ueda et al., 1988; van Netten and Kramer, 1992). *B. cereus* can cause not only food spoilage and decomposition but also food poisoning via its emetic or diarrheal toxins (Crielly et al., 1994; Fangio et al, 2010; Granum and Lund, 1997; Melling and Capel, 1978; Shinagawa et al., 1985; Vilas-Boas et al., 2007). The routine control of contamination by *B. cereus* is an important issue for manufacturers in their food processing and distribution facilities.

Both Mannitol Yolk Polymyxin B agar (MYP) and Kim and Goepfert (KG) agar with NaCl and Glycine agar (NGKG) are used for the detection of *B. cereus* (Mossel et al., 1967; Kim and Goepfert, 1971). However, these methods require the use of egg yolk in preparing the media for determining *B. cereus* contamination and thereby increase labor costs and the time required. Additionally, spreading samples onto this medium is troublesome.

In consideration of these points, the Compact Dry[R] X-BC method (CD-XBC; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) has been developed based on the unique Compact Dry system (Mizuochi and Kodaka, 2000). The CD-XBC system consists of a unique dish, nonwoven fabric, peptone, NaCl, glycine, mannitol, chromogenic substrate, antibiotics and a gelling agent. The plate is incubated at 35℃ for 24 ± 2h (Fig. 1). A one milliliter amount can be inoculated onto the CD-XBC. This method also eliminates the disadvantages of conventional methods since CD-XBC is a pre-sterilized, ready-to-use medium. Glycine and antibiotics inhibit the growth of bacteria, yeasts and molds other than *B. cereus*. Mannitol and chromogenic substrate for α-glucosidase can differentiate *B. cereus* from other bacteria which may grow on the CD-XBC plate.

In this study, the CD-XBC method was compared with conventional methods and its performance in the detection and enumeration of *B. cereus* was evaluated.

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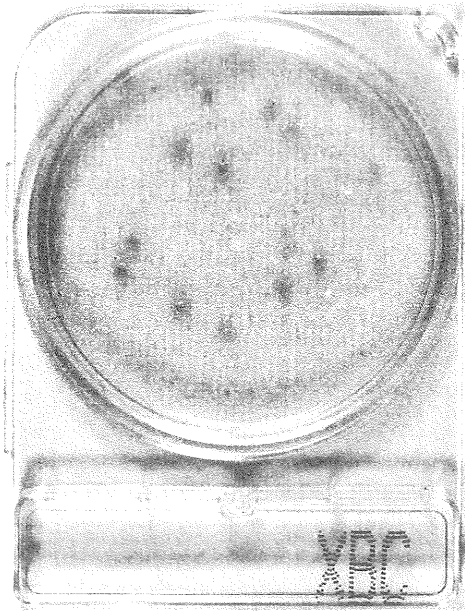

FIG. 1. CDXBC. *B. cereus* ATCC 19637 grew as blue/green colored colonies on CD-XBC after 24h of incubation at 35℃.

Thirteen strains of *B. cereus* were used for the inclusivity study. For the exclusivity study, fifteen *Bacillus* spp. strains other than *B. cereus*, 44 gram-positive strains excluding *Bacillus* spp., 44 gram-negative strains and 3 yeasts were used. Bacterial strains were prepared in Tryptic Soy Broth (Difco, Becton Dickinson, Detroit, MI, USA) at 35℃ for 24h, and yeast strains were cultured in Sabouraud Dextrose Broth (Difco) at 25℃ for 72h. Each culture was diluted serially by saline (0.85% NaCl). One milliliter of each suspension was inoculated onto the CD-XBC. After 24 ± 2h incubation at 35℃, the characteristics and the number of colonies were examined. Inoculated bacterial numbers were confirmed by Tryptic Soy Agar (TSA; Difco) at the same time and under the same conditions.

Results from both the inclusivity and the exclusivity studies are shown in Table 1. All 13 *B. cereus* strains grew as blue/green colored colonies on CD-XBC. Of 15 *Bacillus* spp. strains other than *B. cereus*, 4 strains grew as blue/green colonies, whereas 11 strains did not grow on CD-XBC. All of the 4 blue/green colored colony-forming *Bacillus* spp. were *B. thuringiensis*. Of 44 gram-positive bacteria other than *Bacillus* spp., 3 strains grew as white colonies on CD-XBC, whereas 41 strains failed to grow. Of 44 gram-negative bacteria, 2 strains grew as white colonies, whereas 42 strains failed to grow. The total of 3 yeasts failed to grow.

The CD-XBC method was compared with the MYP (Merck Ltd., Tokyo, Japan) and the NGKG (Nissui Pharmaceutical Co., Ltd.) methods using artificially contaminated food samples. One hundred and thirty samples (58 cooked rice products, 49 dishes of cooked noodles, 9 dishes of cooked gratin and 14 delicatessen samples) were purchased from retail stores. For confirmation that these samples were negative for *B. cereus*, after being homogenized with saline for 2min by a homogenizer (Pro-media SH-001, ELMEX LIMITED, Tokyo, Japan), samples were inoculated onto two plates of MYP and NGKG, and incubated for 24h at 30℃.

For the comparison experiments, spores of three strains of *B. cereus* (ATCC 19637, ATCC 14579, ATCC 11778, American Type Culture Collection, Manassas, VA) were used randomly for inoculation. Each 10g sample was inoculated at the following levels: low (2-3 log CFU/g), medium (3-4 log CFU/g) and high (4-5 log CFU/g). Each artificially contaminated sample was added to a 9-fold volume of saline and was homogenized for 2min. Subsequently, each homogenized sample was subjected to 10-fold serial dilution by saline. Dual measurements were then carried out for each method. One milliliter of each dilution was inoculated onto the CD-XBC plate. After 24h of incubation at 35℃, the blue/green colonies on CD-XBC were determined to be *B. cereus*. For the two other methods, MYP and NGKG were inoculated with 0.1ml of the sample. Samples were then spread onto the surface of each medium with a sterilized bacteria spreader (Nissui Pharmaceutical Co., Ltd.). After 24h of incubation at 30℃, typical colonies were counted. Result s were converted into log CFU of *B. cereus* per gram of each tested food. Statistical analyses were carried out with Microsoft Excel 2000 at the significance level of $P = 0.05$.

The correlation coefficients (r), slopes, intercepts and 95% confidence limits between the CD-XBC and MYP methods, and the CD-XBC and NGKG methods are shown in Table 2. The r values between CD-XBC and MYP, and CD-XBC and NGKG, were 0.972 and 0.971, respectively. The slopes and intercepts of analyzed regression lines for all comparisons were close to 1.00 and 0.00, respectively. No significant difference was shown between CD-XBC and MYP, and CD-XBC and NGKG by one-way ANOVA ($P > 0.05$), respectively.

B. cereus food poisoning is caused by either emetic or diarrheal toxins. The diarrheal toxin is produced during vegetative growth of *B. cereus* in the small intestine whereas the emetic toxin is produced by growing cells in the food (Granum and Lund, 1997). Hence direct detection of *B. cereus* emetic toxins would be effective for the prevention of *B.*

TABLE 1-1. Strains tested for growth and color using CD-XBC (1)

	CD-XBC		TSA
	Color	logCFU/ml	logCFU/ml
Gram positive bacteria			
Bacillus spp.			
B.cereus ATCC 19637	blue/green	7.21	7.03
B.cereus ATCC 11778	blue/green	7.16	7.07
B.cereus ATCC 14579	blue/green	7.32	7.29
B.cereus NS 5109	blue/green	6.89	7.04
B.cereus NS 9116	blue/green	5.82	6.81
B.cereus NS 5201	blue/green	6.87	6.92
B.cereus NS 5202	blue/green	6.56	6.87
B.cereus NS 5203	blue/green	6.72	7.07
B.cereus NS 5204	blue/green	7.48	7.32
B.cereus NS 5205	blue/green	6.20	6.83
B.cereus NS 5206	blue/green	6.20	7.02
B.cereus NS 5207	blue/green	6.86	7.17
B.cereus NS 5208	blue/green	7.12	7.28
B.circulans ATCC 4513	not grown	—	7.96
B.coagulans ATCC 7050	not grown	—	7.66
B.polymyxa ATCC 842	not grown	—	8.19
B.pumilus ATCC 7061	not grown	—	6.62
B.sphaericus ATCC 14577	not grown	—	6.79
B.thuringiensis ATCC 10792	blue/green	6.48	6.63
B.thuringiensis NBRC 3951	blue/green	6.86	7.13
B.thuringiensis NBRC 13865	blue/green	6.31	6.71
B.thuringiensis NBRC 13866	blue/green	6.57	6.72
B.licheniformis ATCC 14580	not grown	—	6.77
B.subtilis ATCC 6633	not grown	—	7.17
B.subtilis NBRC 13719	not grown	—	7.18
B.subtilis NBRC 13721	not grown	—	7.96
B.subtilis NBRC 13722	not grown	—	6.94
Bacillus sp. isolated from natto	not grown	—	7.00
Non-*Bacillus* spp. bacteria			
C.minutissimum ATCC 23348	not grown	—	6.99
C.renale ATCC 19412	not grown	—	7.19
C.xerosis ATCC 373	not grown	—	6.65
E.avium ATCC 14025	not grown	—	7.37
E.durans ATCC 19432	not grown	—	7.44
E.casseliflavus ATCC 51328	not grown	—	8.14
E.faecalis ATCC 19433	not grown	—	8.84
E.faecalis ATCC 29212	not grown	—	8.48
E.faecalis ATCC 49383	not grown	—	8.08
E.faecalis ATCC 49532	not grown	—	8.80
E.faecalis ATCC 51299	not grown	—	8.75
E.faecalis ATCC 51575	not grown	—	8.70
E.faecium ATCC 19434	not grown	—	8.97
E.faecium ATCC 51559	not grown	—	7.77
E.faecium ATCC 700221	not grown	—	8.26
E.gallinarum ATCC 49608	not grown	—	7.72
E.hirae ATCC 8043	not grown	—	8.57
E.mundtii ATCC 43186	not grown	—	8.57
E.raffinosus ATCC 49427	not grown	—	7.31
L.lactis ATCC 12315	not grown	—	7.52
L.citreum JCM 9698	not grown	—	7.59
L.mesenteroides ATCC 27258	not grown	—	8.10
M.luteus ATCC 9341	not grown	—	7.52
P.acidilactici JCM 5885	not grown	—	8.11
S.aureus ATCC 12600	not grown	—	8.37
S.aureus ATCC 25923	not grown	—	8.19
S.aureus ATCC 29213	not grown	—	8.33
S.aureus ATCC 6538	not grown	—	8.15
S.aureus ATCC 6538P	not grown	—	8.13
S.aureus MRSA NS 7167	not grown	—	8.53
S.auricularis ATCC 33753	not grown	—	7.24

continue to Table 1-2

TABLE 1-2. Strains tested for growth and color using CD-XBC (2)

	CD-XBC		TSA
	Color	logCFU/ml	logCFU/ml
continue			
S.capitis ATCC 27840	not grown	—	8.34
S.epidermidis ATCC 12228	not grown	—	7.41
S.epidermidis ATCC 14990	not grown	—	7.71
S.haemolyticus ATCC 29970	not grown	—	7.03
S.hominis ATCC 27844	not grown	—	7.73
S.intermedius ATCC 29663	not grown	—	8.23
S.lentus ATCC 29070	white	5.00	7.04
S.saprophyticus ATCC 15305	not grown	—	7.75
S.sciuri ATCC 29062	white	4.00	7.63
S.simulans ATCC 27848	not grown	—	7.71
S.warneri ATCC 27836	not grown	—	7.38
S.xylosus ATCC 29971	white	3.00	6.32
S.thermophilus ATCC 14485	not grown	—	6.61
Gram negative bacteria			
Aeromonas hydrophila JCM 3976	not grown	—	7.42
Alcaligenes denitrificans JCM 5490	not grown	—	7.88
Alcaligenes xylosoxidans JCM 9659	not grown	—	8.21
Alcaligenes faecalis JCM 1474	not grown	—	7.59
C.amalonaticus ATCC 25405	not grown	—	8.74
C.freundii ATCC 8090	not grown	—	8.72
C.koseri ATCC 25408	not grown	—	8.71
E.aerogenes ATCC 13048	not grown	—	8.76
E.amnigenus ATCC 33072	not grown	—	8.17
E.cloacae ATCC 13047	not grown	—	8.85
E.intermedius ATCC 33423	not grown	—	7.85
E.intermedius ATCC 33110	not grown	—	7.95
E.sakazakii ATCC 29544	not grown	—	8.51
E.blattae JCM 1650	not grown	—	8.59
E.coli ATCC 11775	not grown	—	8.63
E.coli ATCC 25922	not grown	—	8.85
E.coli ATCC 8739	not grown	—	8.75
E.coli ATCC 9637	not grown	—	8.72
E.coli Serotype O157 ATCC 35150	not grown	—	8.81
E.coli Serotype O157 ATCC 43888	not grown	—	8.84
E.fergusonii JCM 5897	not grown	—	8.76
E.fergusonii JCM 5899	not grown	—	8.62
E.hermanii JCM 1473	not grown	—	8.58
H.alvei ATCC 13337	not grown	—	8.21
K.oxytoca ATCC 13182	not grown	—	9.06
K.ozaenae ATCC 11296	not grown	—	9.08
K.pneumoniae ATCC 13883	not grown	—	8.68
K.ascorbata ATCC 33433	not grown	—	8.60
K.cryocrescens ATCC 33435	not grown	—	8.24
M.morganii ATCC 25830	not grown	—	8.97
P.mirabilis ATCC 29906	white	7.00	8.91
P.vulgaris ATCC 13315	not grown	—	8.78
P.aeruginosa ATCC 10145	not grown	—	8.88
P.aeruginosa ATCC 9721	not grown	—	8.06
P.aeruginosa ATCC 27853	not grown	—	8.40
P.aeruginosa ATCC 9027	not grown	—	8.62
P.putida ATCC 12633	not grown	—	8.71
R.aquatilis ATCC 33071	not grown	—	6.08
*S.*Typhimurium ATCC 13311	not grown	—	8.60
*S.*Choleraesuis ATCC 13312	not grown	—	8.63
S.fonticola ATCC 29844	not grown	—	8.56
S.liquefaciens ATCC 27592	not grown	—	8.62
S.marcescens ATCC 13880	white	7.00	8.72
S.marcescens ATCC 8100	not grown	—	8.74
Yeasts			
C.albicans ATCC 2091	not grown	—	6.02
C.albicans ATCC 10231	not grown	—	6.71
S.cerevisiae ATCC 9080	not grown	—	6.45

[a] Standard strains were derived from ATCC (American Type Culture Collection), JCM (Japan Collection of Microorganisms) and NBRC (NITE Biological Resource Center, Japan).
[b] NS strains were isolated from clinical specimens.
[c] Recovered bacterial number is represented as logCFU/ml.

Table 1 represents results of inclusivity and exclusivity studies for CD-XBC.
Each strain was cultured on CD-XBC for 24 ± 2h at 35℃.

TABLE 2. Correlation with two conventional methods for enumerating *B. cereus* in artificially contaminated food samples[a,b,c]

Parameter	CD-XBC vs. MYP	CD-XBC vs. NGKG
No. of samples	130	130
Correlation coefficient (*r*)	0.972	0.971
Slope	1.04	0.94
Intercept	−0.25	0.11
95% confidence limits	±0.15	±0.16
ANOVA (*P*)[d]	0.32	0.31

[a] Artificially contaminated food samples were inoculated with spores of *B. cereus* ATCC19637, ATCC14579 or ATCC11778 randomly.

[b] Each strain was inoculated at high(4-5 logCFU/g), medium(3-4 logCFU/g) or low(2-3 logCFU/g) bacterial levels randomly.

[c] Fifty-eight cooked rice products, 49 dishes of cooked noodles, 9 dishes of cooked gratin and 14 delicatessen products were used as artificially contaminated food samples, respectively.

[d] ANOVA (one way analysis of variance) was performed at a significance level of 0.05.
$P<0.05$ is a statistically significant difference between two methods.

Table 2 represents the correlation between CD-XBC and each method in the examination of artificially contaminated food. After the counts (CFU/g) from artificially contaminated food samples were converted to logarithm (logCFU/g) values, linear regression equations for CD-XBC with respect to each method were calculated.

cereus food poisoning. For the detection of emetic toxins, cytoplasmic vacuolating bioassay for Hep-2 cell (Hughes et al., 1988) or mass spectrograph with HPLC (Haggblom et al., 2002) should be used because emetic toxins show poor antigenicity (Melling and Capel, 1978). Moreover, for the detection of diarrheal toxins, the reverse passive latex immunoassay (Beecher and Wong, 1994; Schoeni and Wong, 2005) has been developed. The methods for the direct detection of these toxins, require special devices, skills and have high costs. Hence, it is difficult to apply these methods for the detection of toxins in daily hygiene control.

For monitoring *B. cereus* directly, PCR-based methods (Martinez-Blanch et al., 2010; Wehrle et al., 2009; Yabutani et al., 2009) and chromogenic culture methods (Fricker et al., 2008; Peng et al., 2001; Reissbrdt et al., 2004) have been developed. The molecular methods, such as the PCR-based methods, can detect *B. cereus* rapidly with precision and accuracy but they also require specific devices, skills and have high costs. The culture methods do not have the ability to differentiate completely *B. cereus* from *B. thuringiensis*, a member of the cereus group, because *B. thuringiensis* has quite the same characteristics as *B. cereus* except for producing parasporal crystals (Vilas-Boas et al., 2007). On the other hand, the chromogenic culture methods, including the CD-XBC, make operations more cost-effective, and detect *B. cereus* comparatively easily and rapidly because these methods do not use egg yolk. The formulation of CD-XBC is especially cost-effective because its chromogenic substrate for α-glucosidase is less costly and can be more easily handled than the enzyme substrate for phosphatidylinositol phospholipase C (PI-PLC) that is used for several *B. cereus* chromogenic media. Moreover the CD-XBC method has both high selectivity and a good correlation with conventional methods. Therefore the simplicity of using CD-XBC and the results here suggest the CD-XBC method is a suitable alternative for the detection and enumeration of *B. cereus* in daily food hygiene control.

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REFERENCES

Beecher, D. J. and Wong, A. C. L. (1994) Identification and analysis of the antigens detected by two commercial *Bacillus cereus* diarrheal enterotoxin immunoassay kits. *Appl. Environ. Microbiol.*, **60**, 4614-4616.

Crielly, E. M., Logan, N. A. and Anderton, A. (1994) Studies on the *Bacillus* flora of milk and milk products. *J. Appl. Bacteriol*,, **77**, 256-263.

Fangio, M. F., Roura, S. I. and Fritz, R. (2010) Isolation and identification of *Bacillus* spp. and related genera from different starchy foods. *J. Food Sci.*, **75**, 218-229.

Fricker, M., Reissbrodt, R. and Ehling-Schulz, M. (2008) Evaluation of standard and new chromogenic selective plating media for isolation and identification of *Bacillus cereus*. *Int. J. Food Microbiol.*, **121**, 27-34.

Granum, P. E. and Lund, T. (1997) *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Lett.*, **157**, 223-228.

Haggblom, M. M., Apetroaie, C., Andersson, M. A. and Salkinoja-Salonen, M. S. (2002) Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under various conditions. *Appl. Environ. Microbiol.*, **68**, 2479-2483.

Hughes, S., Bartholomew, B., Hardy, J. C. and Kramer, J. M. (1988) Potential application of a HEp-2 cell assay in the investigation of *Bacillus cereus* emetic-syndrome food poisoning. *FEMS Microbiol. Lett.*, **52**, 7-12.

Kim H. U. and Goepfert J. M. (1971) Enumeration and

identification of *Bacillus cereus* in foods. *Appl. Microbiol.*, **22**, 581-587.

Martinez-Blanch, J. F., Sachez, G., Garay, E. and Aznar, R. (2010) Evaluation of a real-time PCR assay for the detection and quantification of *Bacillus cereus* group spores in food. *J. Food Prot.*, **73**, 1480-1485.

Melling, J. and Capel, B. J. (1978) Characteristics of *Bacillus cereus* emetic toxin. *FEMS Microbiol. Lett.*, **4**, 133-135.

Mizuochi, S. and Kodaka, H. (2000) Evaluation of dry sheet medium culture plate (Compactdry TC) method for determining numbers of bacteria in food samples. *J. Food Prot.,* **63**, 665-667.

Mossel, D. A. A., Koopman M. J. and Jongerius E. (1967) Enumeration of *Bacillus cereus* in foods. *Appl. Microbiol.*, **15**, 650-653.

Peng. H., Ford, V., Frampton, E. W., Restaion, L., Shelef, L. A. and Spitz, H. (2001) Isolation and enumeration of *Bacillus cereus* from foods on a novel chromogenic plating medium. *Food. Microbiol.*, **18**, 231-238.

Reissbrodt, R., Raβbach, A., Burghardt, B., Rienacker, I., Mietke, H., Schleif, J., Tschape, H., Lyte, M. and Williams P. H. (2004) Assessment of a new selective chromogenic *Bacillus cereus* group plating medium and use of enterobacterial autoinducer of growth for cultural identification of *Bacillus* species. *J. Clinical Microbiol.*, **42**, 3795-3798.

Schoeni, J. L. and Wong, A. C. L. (2005) *Bacillus cereus* food poisoning and its toxins. *J. Food Prot.*, **68**, 636-648.

Shinagawa, K., Matsusaka, N., Konuma, H. and Kurata, H. (1985) The relation between the diarrheal and other biological activities of *Bacillus cereus* involved in food poisoning outbreaks. *Jpn. J. Vet. Sci.*, **47**, 557-565.

Ueda, S., Katube, Y. and Kuwabara Y. (1985) Studies on the ecology of *Bacillus cereus* (1). The biochemical characteristics of *B. cereus* strains isolated from food, food poisoning outbreaks and environment. *J. Antibact. Antifung. Agents*, **13**, 547-554 (in Japanese).

Ueda, S. and Kuwabara Y. (1988) The ecological study of *Bacillus cereus* in rice crop processing (2). On the contamination of *B. cereus* in unhulled, unpolished and polished rice during the process of rice. *J. Antibact. Antifung. Agents*, **16**, 115-120 (in Japanese).

Ueda, S., Yamazaki, M., Machida, A., Akao, A. and Kuwabara Y. (1988) The ecological study of *Bacillus cereus* in rice crop processing (3). -Airborne *B. cereus* in rice mills-. *J. Antibact. Antifung. Agents*, **16**, 459-464 (in Japanese).

van Netten, P. and Kramer, J. M. (1992) Media for the detection and enumeration of *Bacillus cereus* in foods: a review. *Int. J. Food Microbiol.*, **17**, 85-99.

Vilas-Boas, G. T., Peruca, A. P. S. and Arantes, O. M. N. (2007) Biology and taxonomy of *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. *Can. J. Microbiol.*, **53**, 673-687.

Wehrle, E., Moravek, M. Dietrich, R., Burk, C., Didier, A. and Martlbauer, E. (2009) Comparison of multiplex PCR, enzyme immunoassay and cell culture methods for the detection of enterotoxinogenic *Bacillus cereus. J. Microbiol. Methods*, **78**, 265-270.

Yabutani, M., Agata, N. and Ohta, M. (2009) A new rapid and sensitive detection method for cereulide-producing *Bacillus cereus* using a cycleave real-time PCR. *Lett. Appl. Microbiol.*, **48**, 698-704.