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A comparison of ZEN double-quenched probe and SYBR GreenER chemistries in the real-time PCR based quantitative detection of enterotoxigenic *Bacillus cereus* in milk

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ABSTRACT

Aims: Comparison between ZEN™ double-quenched probe and SYBR GreenER™ real-time PCR assay to develop a sensitive and specific assay for the direct detection and quantification of enterotoxigenic *Bacillus cereus* in milk.
Methodology and results: Novel primers and probe were designed to target the enterotoxigenic *nhe* gene. The performance of ZEN™ double-quenched probe and SYBR GreenER™ chemistry were compared by using known concentrations of purified DNA. ZEN™ double-quenched probe showed a dynamic range of 3 log units and sensitivity of 600 fg/reaction or 100 copies/reaction. SYBR GreenER™ chemistry had a wider quantitative dynamic range of 6 log units with sensitivity down to 6 fg/reaction or 1 copy number/reaction. Thus, SYBR GreenER™ chemistry was 100× more sensitive with wider quantification range compared to ZEN™ probe chemistry. Similar result was also found for SYBR GreenER™ assay and ZEN™ probe chemistry in DNA extracted directly from artificially inoculated milk, with the lowest limit of detection by SYBR GreenER™ assay in the range of 6 fg/reaction or 25 copies/mL and it quantified *Bacillus cereus* in milk with high relative accuracy.
Conclusion: SYBR GreenER™ assay provides a fast, sensitive and specific detection and quantification of enterotoxigenic *Bacillus cereus* and allowed a direct assessment and quantification of *Bacillus cereus* from milk food sample.
Conclusion, significance and impact of study: The study shows an efficient, specific and highly sensitive method of directly assessing the enterotoxigenic *Bacillus cereus* from milk product, using cheaper dsDNA binding SYBR GreenER™ dye.

Keywords: Real-time PCR, *Bacillus cereus*, DNA, Bacteria, ZEN™ double-quenched probe, SYBR GreenER™

INTRODUCTION

Bacillus cereus is an opportunistic foodborne pathogen, which causes either diarrhoea or emetic syndrome (Kontiranta *et al.*, 2000; Senesi and Ghelardi, 2010). The diarrhoeal syndrome is characterized by diarrhoea, nausea without vomiting and abdominal pain, which occurs 8 to 16 h after the exposure (Rajkowski and Bennett, 2003). This syndrome is generally associated with three toxins*: Haemolysin BL, Non-Haemolytic Enterotoxin* (*nhe*) and *Cytotoxin K* (Beecher *et al*., 1995; Lund *et al*., 2000; Fagerlund *et al*., 2008). Unlike diarrhoeal syndrome, the emetic syndrome causes vomiting with occasional diarrhoea (Rajkowski and Bennett, 2003). The emetic symptoms typically occur 1 to 5 h after the consumption of food containing the emetic toxin, cereulide, which is encoded by the *ces* gene of *Bacillus cereus* (Granum and Lund, 1997; Rajkowski and Bennett, 2003). However, proteins and enzymes such as phosphatidylinositol-specific phospholipase, phosphatidylcholine specific phospholipases C, sphingomyelinase, cereolysin O, enterotoxin FM, haemolysin II and haemolysin III have also been suspected to contribute to pathogenicity of *Bacillus cereus* (Stenfors Arnesen *et al*., 2008; Ceuppens *et al*., 2011).

This bacterium is ubiquitous in different types of environments including but not limited to soil, dust, sediments, water, plants (Kramer and Gilbert, 1989; Kontiranta *et al*., 2000; Vilain *et al*., 2006; Ribeiro *et al*., 2010) as it forms endospores that can withstand

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temperature extremes and other harsh environmental conditions (Kontiranta *et al*., 2000). The spores can even survive a temperature of 100 °C (Anonymous, 1996; Kontiranta *et al*., 2000). Endurance to such high temperature allows the bacteria to survive adverse conditions followed by germination at more favourable conditions, optimally at 25-37 °C (Drobniewski, 1993). Some strains are also capable of spreading at high (75 °C) and low (3 °C) temperatures (Drobniewski, 1993). Consequently, *Bacillus cereus* also occurs in a variety of food products including pasteurized milk, infant food, meat products and eggs (Colmer, 1948; Becker *et al*., 1994; Te Giffel *et al*., 1997; Nortjé *et al*., 1999).

Bacillus cereus is typically detected by culture-based methods. It is a very labour intensive and slow method, needing at least 36 h of incubation for isolation, enumeration and identifications (Tallent *et al*., 2012). Real-time PCR is an alternative quantitative approach for the detection of *Bacillus cereus* with possibility of automation. It uses either unspecific double-stranded DNA (dsDNA) binding dyes (eg: SYBR Green, PicoGreen, EvaGreen, etc) or probe-based chemistries (eg: TaqMan probe, scorpion probes, etc.). New dyes and probes are designed continually to further improve sensitivity and accuracy of the method (Salihah *et al*., 2016). Probe-based chemistry for real-time PCR offers higher specificity than dsDNA binding dyes though it is more expensive and more difficult to design. It requires the binding of primers and probes to the DNA sample for fluorescent dyes to be observable. In contrast, unspecific dsDNA binding dyes offer cheaper and simpler alternative to probe-based chemistry, as it does not require additional probe design. However, unspecific dsDNA binding dye chemistry binds and fluoresce with any dsDNA, thus each newly optimize assay requires further analysis to ensure that the observed fluorescence amplification is from specific target sequence but not from unspecific amplification (i.e. primer-dimer) (Salihah *et al.*, 2016). As real-time PCR instruments advances, melting curve analysis and High-Resolution Melting (HRM) analysis have further simplified the process, as post-PCR analysis (i.e.: for unspecific amplifications) can be directly done *in-situ* after amplifications, without additional reagents or preparation and keeping PCR samples inside the real-time PCR instruments. Melting curve analysis and/or HRM provided an alternative to gel electrophoresis, as it allows differentiation of amplified target sequence and unspecific amplification sequences based on the melting points of the sequences.

This study aimed to develop a novel and sensitive real-time PCR assay by assessing two different real-time PCR fluorescence strategies, i.e. ZEN™ double-quenched probe and improved SYBR Green dye – a proprietary dye from ThermoFisher Scientific – to improve the sensitivity of direct detection and quantification of enterotoxigenic *Bacillus cereus* in food specifically whole milk. ZEN™ is a new probe system by Integrated DNA Technologies (IDT, Coralville, USA) that increases sensitivity and precision while allowing longer probes design. It has an internal quencher inserted between the reporter and quencher to doubly-quench the reporter signal while keeping the background signal low. Whereas, SYBR GreenER™ is an improvement over the SYBR Green dye I from ThermoFisher Scientific that addresses limitations of SYBR Green dyes, such as PCR inhibitions, lower sensitivities and fluorescence. SYBR GreenER™ can be used with all real-time PCR systems, which are set to detect SYBR Green I fluorescence without re-calibrations.

MATERIALS AND METHODS

Genomic DNA of bacterial strains

The genomic DNA used in this study were obtained from American Type Culture Collection (ATCC, Manassas, USA) as listed in Table 1 for exclusivity and inclusivity analyses. The concentration and purity of the genomic DNA were measured by Spectrophotometric method on NanoPhotometer™ P-Class (Implen, Munchen, Germany).

Table 1: Genomic DNA of bacteria strains from ATCC.

Bacteria	Strain no./ATCC no.
Bacillus cereus	ATCC 14579
Staphylococcus aureus	ATCC 25923
Legionella pneumophila	ATCC 33152
Bacillus subtilis	ATCC 23857
Salmonella enterica	ATCC 13311
Escherichia coli	ATCC 35401
Clostridium pefringens	ATCC 13124
Shigella flexneri	ATCC 29903
Campylobacter jejuni	ATCC 33292
Yersinia enterocolitica	ATCC 27739
Aeromonas hydrophila	ATCC 7966
Plesiomonas shigelloides	ATCC 51903
Streptococcus pyogenes	ATCC 19615
Cronobacter sakazakii	ATCC BAA-894
Mycobacterium avium	ATCC BAA-968

Bacterial strain, culture media, growth method and cell counting

The bacteria strain ATCC 14579 was bought from Microbiologics, Inc (Minnesota, USA). It was cultured in brain heart infusion (BHI) broth at 30 °C for 48 h. The total cell count of the culture was determined with a Neubauer haemocytometer (Hausser Scientific, Horsham, USA).

Primers and probes designed

The primers and probe as listed in Table 2 were designed targeting the *nhe* gene (Granum and Lund, 1997; Granum *et al*., 1999) by using the PrimerQuest Tool (IDT). Primer-Blast (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) and OligoAnalyzer Tool (IDT) confirmed *in silico* that the primer pairs and probes were free from strong secondary structures (i.e primer dimers and hairpin structures) and they recognized only the target bacteria.

Table 2: Probes and primers designed in present study to quantitative analyse *Bacillus cereus*.

Gene	GenBank accession no.	Primer	Oligonucleotide sequence (5'-3')[a]	Position	Product size (bp)
nheA	Y19005	WnheF	CATTAAGGTAAATGCGATGAGTAGC	1079-1255	176
		WnheR	CGTTTCCTGCTAGTTCATAGAGC		
		WnheP	6-FAM/CAAAGGCGA/ZEN/ATGTGCGAGAGTGGA/IB®FQ		

Zen™ double-quenched probe Real-time PCR

The assay was carried out on the Applied Biosystem® 7500 real-time PCR system (USA) in a 25 μL PCR mixture that contained Millipore water, 1× of Buffer II (Applied Biosystem™ Lifetechnologies, Van Allen Way, USA), 500 nM of both the forward and reverse primers, 250 nM of the probe, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (Invitrogen™ Lifetechnologies, Van Allen Way, USA), 0.1× ROX reference dye (Invitrogen™ Lifetechnologies), 0.625 U of AmpliTaq DNA polymerase (Applied Biosystem™ Lifetechnologies, Van Allen Way, USA) and 4 μL of DNA template.

Amplification was conducted as follows: initial denaturation at 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 15 sec, Annealing for 15 sec at 58 °C, and extension phase at 72 °C for 1 min. Positive and negative controls were added for each assay. Approximately 1×10^6 fg or 4×10^6 fg of *Bacillus cereus* ATCC 14579 genomic DNA were used as template for positive controls. Water was used as template for negative controls.

SYBR® GreenER™ Real-time PCR

The SYBR Green real-time PCR assay was performed by using the Applied Biosystem® 7500 real-time PCR system with the SYBR® SELECT Mastermix by Lifetech. Concentrations of 1× SYBR® SELECT mastermix, 0.25 μM of both the forward and reverse primers, Millipore water, in a final volume of 20 μL including 4 μL of DNA template.

The three-step protocol was performed as follows: UDG activation at 50 °C for 2 min, initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, annealing for 15 sec at 58 °C, and extension phase of 72 °C for 1 min. Positive and negative controls were added for each assay. Approximately 1×10^6 fg or 4×10^6 fg of *Bacillus cereus* ATCC 14579 genomic DNA were used as template for positive controls. Water was used as template for negative controls.

Specificity of assays

The specificity of both assays was evaluated against the bacterial species listed in Table 1 by using 4×10^6 fg/reaction of bacterial DNA. One copy of *B. cereus* strain ATCC 14579 is approximately equivalent to 6 fg of genomic DNA (Ivanova *et al*., 2003).

Performance validations

The performance of selected assays was evaluated by analyzing standard curves, which were generated from 10-fold dilutions of purified genomic DNA of *Bacillus cereus* ATCC 14579 in the range of 10 to 1×10^7 fg/reaction.

Artificial inoculation of milk

The sensitivity of the SYBR® GreenER™ dye assay was determined with DNA extracted from inoculated milk. Sterile whole milk was contaminated with serial dilutions of *Bacillus cereus* ATCC 14579. The DNA was extracted from the milk matrix by using the adapted DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany). Genomic DNA from food matrix was extracted by a combination of boiling method and DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany). The protocol was modified as follows: 400 μL of the sample was centrifuged for 30 min at 21,000 *g*. The pellet was washed twice with 500 μL of 1× TE buffer (pH 8.0). The pellets were then re-suspended in 200 μL of 1× TE buffer and incubated at 99 °C for 15 min. The supernatant was then lysed with 200 μL AL and 25 μL Qiagen Proteinase K at 70 °C for 30 min. After heating, the rest of manufacturer's protocol from DNA extraction of Gram-positive bacteria was followed to the DNA elution step. DNA was eluted once from the column with 40 μL AE buffer. Finally, 4 μL of extracted DNA was used as DNA template for SYBR® GreenER™ dye assay.

RESULTS AND DISCUSSION

Design and specificity of assay

In this study, the primer and ZEN™ double-quenched probe were designed using the PrimerQuest Tool (IDT) for the detection and quantification of the enterotoxigenic gene *nhe.* The *nhe* gene was chosen as target due to its wider distribution in *B. cereus* strains when compared to *hbl* and *cytK* genes (Stenfors Arnesen *et al*., 2008; Ankolekar *et al*., 2009; Martínez-Blanch *et al*., 2009) and its presence as a single copy gene allows for direct quantification of enterotoxigenic *B. cereus*. The *nhe* gene encodes three non-haemolytic enterotoxin components - A, B and C (Granum *et al.*, 1999). All three components are required for cytotoxic activity (Lindbäck *et al*., 2004) as explained in the enterotoxigenic mechanism of the *nhe* toxins by Fagerlund et al. (Fagerlund *et al*., 2008).

The specificity of the WnheF/R SYBR® GreenER™ dye assay and WnheF/R/P ZEN™ double-quenched probe were determined experimentally using 4 × 10^6 fg/reaction of species listed in Table 1. No amplification of non-*Bacillus cereus* bacteria was observed for both the assays. Post-PCR melting curve analysis for the SYBR® GreenER™ assay showed only a single peak for the positive control (*B. cereus* ATCC 14579). This result corroborated with post-PCR gel-electrophoresis analysis (data not shown). The same result was also observed for ZEN™ double-quenched probe assay. Thus, both assays were highly specific and suitable for detection and quantification of *B. cereus.*

However it should be noted that the primer pair and probe designed were shown in-silico to also target enterotoxigenic *B. cereus* group (i.e: *B. anthracis, B. thuringiensis, B. weihenstephanesis, and B. mycoides*), which may also exhibit these enterotoxigenic genes (Hsieh *et al*., 1999; Hendriksen *et al*., 2006) and have been implicated in food poisoning outbreaks (McIntyre *et al.*, 2008). Thus, these primers could be also used to detect the other enterotoxigenic *B. cereus* group.

Performance validations and quantification limits

The performance of two types of detection chemistry were evaluated, mainly ZEN™ double-quenched probe and SYBR® GreenER™ dye. This was done by analyzing the standard curves and determining the sensitivity of each assay. The standard curves were generated from 10-fold dilutions of *B. cereus* ATCC14579 genomic DNA in the range of 10 to 1 × 10^7 fg per reaction) (Figure 1). Efficiency was calculated from the standard curves by using the equation described by Klein *et al.* (1999). The SYBR® GreenER™ dye assay showed 90.5% efficiency (R^2=0.997) compared to 96.7% (R^2=0.999) for the ZEN™ double-quenched probe assay which are within the recommended efficiency (90 to 110%) and R^2 > 0.99 for real-time PCR.

ZEN™ double-quenched probe assay showed lower linear quantification range (Table 3) of 1 × 10^4 to 1 × 10^7 fg/reaction. It has a higher limit of detection of 6 × 10^2 fg/reaction or 100 copy/reaction extrapolated from three separate real-time PCR runs, On the other hand, SYBR× GreenER™ dye assay showed higher sensitivity of 6 fg/reaction or 1 copy/reaction, with probability rate of 33.333% concluded from three independent experiments. It also has with wider dynamic quantification range, from 10 to 1 × 10^7 fg/reaction (Table 3). Both of the assays were highly reproducible with mean intra- and inter-assay variation (CV%) of approximately 1% and less than 2%, respectively (Table 3).

Thus, it was observed that SYBR® GreenER™ dye based assay performed better than the ZEN™ double-quenched probe-based assay. The novel primers in combination with the SYBR® GreenER™ dye produce highly specific assay without any non-specific binding (i.e: primer-dimer) and had higher sensitivity (6 fg/reaction) compared to ZEN™ double-quenched probe and previous

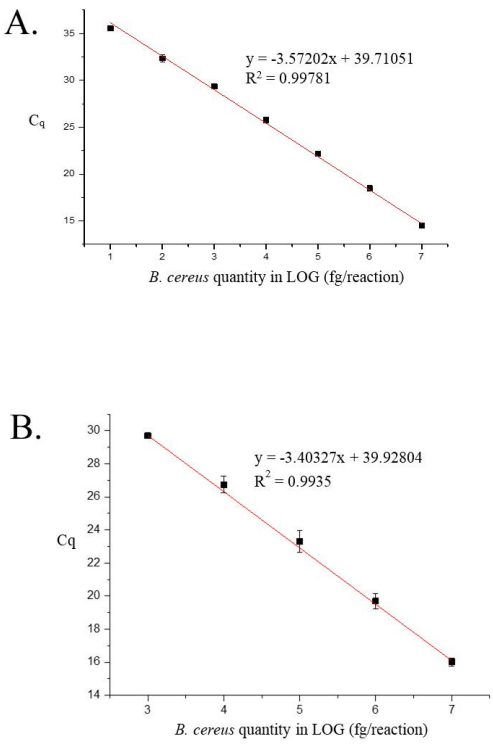

Figure 1: Standard curve of WnheF/R primer pair (A) SYBR® GreenER™ (B) ZEN™ double-quenched probe constructed from the C_q values for 10-fold dilutions of *B. cereus*.

study by Martínez-Blanch *et al*. (2009), that had reported sensitivity of 12 fg/reaction.

It also had wider quantification range of 6 log units in comparison to ZEN™ double-quenched probe with 3 log units of quantification range. Our result supported Josefsen et al. (2012) in that dsDNA binding dyes, such as SYBR® GreenER™ dye, yield higher fluorescence signal due to higher proportion of dsDNA binding dyes per amplicon compared to 'single signal per amplicon' of the probe based chemistry. Consequently, SYBR® GreenER™ assay allowed faster detection with higher sensitivity and wider quantification range while being comparatively cheaper than probe-based chemistries.

Artificial inoculation of milk

The suitability of both SYBR® GreenER™ assay and ZEN™ double-quenched probe-based assay in detecting bacteria in food sample were further evaluated with full fat milk that were artificially contaminated with serial dilutions of *B. cereus* ATCC 14579 cells. The DNA was directly extracted from the milk samples with a modified DNeasy Blood and Tissue kit by combining it with the boiling method that requires no additional expensive lysozyme enzymatic lysis.

Table 3: Ratio of positive reaction and inter- and intra-assay coefficient variation (CV%) for the WnheF/R/P qPCR assays within the range of 10 to 1 × 10^7 fg of *B. cereus* DNA dilutions for both ZEN™ double-quenched probes and SYBR® GreenER™ chemistry.

Assay	fg/reaction	Ratio of positive reactions[a]	Mean CV% ± SD[b]	
			Intra-assay	Inter-assay
SYBR® GreenER™	1 × 10^7	9/9	0.79 ± 0.613	1.06 ± 0.837
	1 × 10^6	9/9		
	1 × 10^5	9/9		
	1 × 10^4	9/9		
	1 × 10^3	9/9		
	1 × 10^2	9/9		
	10	5/9		
ZEN™ double-quenched probes	1 × 10^7	9/9	1.053 ± 0.234	1.692 ± 0.619
	1 × 10^6	9/9		
	1 × 10^5	9/9		
	1 × 10^4	7/9		
	1 × 10^3	6/9		
	1 × 10^2	0/9		
	10	0/9		

[a] number of positive result per 9 individual reactions
[b] SD is standard deviations

Table 4: *Bacillus cereus* quantification by WnheF/R SYBR® GreenER™ assays in artificially contaminated milk and relative accuracy in comparison to DNA dilutions standard curves.

Assay	fg/reaction[a]	Ratio of positive reactions[b]	Estimated no. ± SD[c]	Relative accuracy (%)
SYBR® GreenER™	6 × 10^5	3/3	8.1239 × 10^4 ± 631.044	81.239
	6 × 10^4	3/3	9.598 × 10^3 ± 252.986	95.982
	6 × 10^3	3/3	9.90 × 10^2 ± 14.133	99.027
	6 × 10^2	3/3	63.487 ± 4.51	63.487
	6 × 10^1	3/3	12.885 ± 0.816	128.851
	6	3/3	--[d]	--[d]
ZEN™ double-quenched probes	6 × 10^5	3/3	6.77745 × 10^5 ± 153.824	112
	6 × 10^4	3/3	--[d]	--[d]
	6 × 10^3	3/3	--[d]	--[d]
	6 × 10^2	1/3	--[d]	--[d]
	6 × 10^1	0/3	--[d]	--[d]
	6	0/3	--[d]	--[d]

[a] Approximate quantity of genomic DNA (fg/reaction) of *Bacillus cereus* ATCC 14579
[b] Number of positive results per reactions
[c] Estimated quantity of *Bacillus cereus* in milk (fg/reaction) based on DNA dilutions standard curve
[d] Values below limit of quantification

For the detection of *B. cereus* in artificially inoculated milk, both the SYBR® GreenER™ assay and ZEN™ double-quenched probe-based assay showed similar sensitivities as with the standard genomic DNA dilutions. The limit of detection for ZEN™ double-quenched probe-based assay was 6 × 10^2 fg/reaction of real-time PCR assay or 1.5 × 10^4 fg of *B. cereus* cells in 1 mL of artificially inoculated milk without pre-enrichment step. In contrast, the SYBR® GreenER™ assay was able to detect as low as 6 fg/reaction or 1.5 × 10^2 fg of *B. cereus* in 1 mL of artificially inoculated milk. This is lower than previously reported studies without the need for pre-enrichment step (Fricker *et al.*, 2007; Martínez-Blanch *et al*., 2009; Wehrle *et al.*, 2010; Fernández-No *et al*., 2011; Dzieciol *et al.*, 2013; Ueda *et al.*, 2013;). This proves that the modified method of isolation in combination with the real-time PCR SYBR® GreenER™ assay proposed was able to lyse the Gram-positive bacteria completely and remove the PCR inhibitors in the highly inhibitory whole milk for successful and sensitive amplifications.

The accuracy of the quantification of inoculated milk was evaluated by comparing the values extrapolated from the standard curve generated from DNA dilution standards (Table 4). ZEN™ double-quenched probe-based assay, only a single concentration of *B. cereus* inoculation in milk (6×10^5 fg/reaction) was quantified with the relative accuracy of 112% (Table 4). This was because of the low quantification range of only 3 log units of ZEN™ double-quenched probe-based assay (Table 3). Whereas for the SYBR® GreenER™ assay, the quantification was relatively accurate for high bacterial contaminations of milk (6×10^3 to 6×10^5 fg/reaction) with accuracy between 81 to 99%. However, lower accuracy is observed for quantification of low bacterial contaminations of milk, relative accuracy for 63.5 and 128%, respectively for 6×10^3 and 6×10^2 fg/reaction. This poor accuracy at low concentration can be attributed to the highly complex matrix of milk, which contains high amount of PCR inhibitors such fat. This can affect the accuracy of quantification of lower amounts of *B. cereus* contaminants in milk.

In conclusion, we have developed a novel, highly sensitive, accurate, comparatively cheaper SYBR® GreenER™ dye real-time PCR assay based on the enterotoxigenic *nhe* gene for fast and direct detection with quantification of *B. cereus.* The method does not require pre-enrichment, and can directly detect and reliably quantify *B. cereus* in the milk by using modified DNeasy Blood and Tissue kit.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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