# Fast EvaGreen Real-time Duplex PCR for the Individual Detection of *Staphylococcus aureus* and *Bacillus cereus* using a Uniform Amplification Strategy

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Abstract: Reliable and sensitive detection of *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) is needed to limit the outbreak of food poisoning thereof. This paper reports the development of two individual duplex real-time PCR assays with subsequent melting curve analyses based on EvaGreen® dye for dual gene detections of two bacteria under uniform amplification condition. The duplex assays targeted thermostable *nuclease* gene (*nuc*) and *heat-shock protein* gene (*htrA*) of *S. aureus* and *non-haemolytic enterotoxin* gene (*nhe*) and *cereolysin A* gene (*cerA*) for *B. cereus* detection. The assays successfully detected both the species with high specificity and sensitivity in genomic DNA samples and in simulated real milk samples. The selectivity was also confirmed against a wide range of background microflora. Sensitivity of 500 cell/mL and 25 cell/mL of milk was obtained respectively for *S. aureus* and *B. cereus*. The proposed methodology allowed for fast, inexpensive, selective and sensitive multi-targets detections of both bacteria in a single amplification run on multiple genes to detect *S. aureus* and *B. cereus* in milk product by using dsDNA binding EvaGreen® dye.



## 1 INTRODUCTION

*S. aureus* and *B. cereus* – prevalent foodborne pathogens – cause overlapping symptoms of diarrhoea, vomiting, and abdominal pains (Bennett and Monday, 2003; Rajkowski and Bennett, 2003) and are potent to become epidemic. Culture-based techniques, while inexpensive in confirming the presence of these pathogens, are labourious and time consuming – taking up to 5 or 8 days respectively for *S. aureus* and *B. cereus* (Bennett and Lancette, 2012; Tallent et al., 2012). It may also help pathogens to reenter the environment because of false negative results of viable but non-culturable pathogens that retained their virulence (Gunasekera et al., 2002).

In recent years, alternate bacterial pathogens detection techniques are developed to overcome the issues presented by conventional culture-based method such electrochemical, biosensor and real-time PCR based detections (Ahmed et al., 2013; Safavieh et al., 2012; 2013; 2014a; 2014b; Salihah et al., 2018; 2019; Tlili et al., 2013; Tolba et al., 2012). Real-time PCR-based detection of infectious agents, especially bacterial enteric pathogens, is becoming popular over conventional culture-based and even gel-based PCR techniques due to higher sensitivity, shorter turnaround time, and enhanced environmental and analyst safety. Faster detection is crucial to identify the source to limit the spread of pathogens while delays in detection may lead to their outbreaks. The higher sensitivity of PCR-based detection reduces the need for pre-enrichment or enrichment of bacteria and limits the possibilities of re-introduction of the bacterial pathogens into the environment (Martínez-Blanch et al., 2009; Salihah et al., 2018; 2019).

However, PCR-based detection based on a single marker gene has limited scope in identifying bacterial pathogens because of the varying occurrence of genes in different strains of targeted bacterial species (Stenfors Aresen et al., 2008; Guinebretiére et al., 2010) as well as due to the variations or deletions of a marker gene at primer-binding sites (Klaassen et al., 2003), which may produce false-negatives results.



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Detecting more than a single gene marker can address this. However, detection of multiple gene markers individually requires more PCR consumables, samples and time. On the other hand, multiplex reactions allow the amplification of two or more genes in a single tube with less amount of consumables and samples. As with any real-time PCR assays, multiplex real-time PCR reactions utilize either sequence specific probe-based or the non-specific dsDNA binding dyes fluorescence detection chemistries (Klaassen et al., 2003). While probe-based chemistry is more specific and allow for quantitative multiplex analysis, it is also more expensive and difficult to design (Salihah et al., 2016). In contrary, the non-specific dsDNA (double-stranded DNA) binding dyes offer a cheaper alternative for multiplex reactions. However, due to their unspecific affinity to any dsDNA, a post-amplification melting curve analysis is required to differentiate different targets' amplicons which makes multiplexing possible (Postollec et al., 2011; Salihah et al., 2016).

This report describes the development of two multiplex assays targeting two gene markers each for *S. aureus* and *B. cereus* by using the EvaGreen® dye chemistry. EvaGreen® dsDNA binding dye was selected because it produces higher melting curve resolution and unlike SYBR Green I, it does not bind preferentially to GC-rich amplicons which can adversely affect the multiplexing detection (Hu et al., 2014; Giglio et al., 2003; Eischeid et al., 2011).

However, as a dsDNA binding dye, EvaGreen® fluoresce in the presence of any dsDNA, giving off the same fluorescent signal. Therefore, all the amplicons of multi-targets real-time PCR amplification are differentiated by mean of the amplicons' unique $T_m$. Several studies have proven that multiplexed real-time PCR with dsDNA binding dyes are possible by differentiating the amplicons of the different targets by their $T_m$ values (Hu et al., 2014; Safdar et al., 2015) which can be obtained immediately and automatically after amplification with zero additional handling with the current real-time PCR instruments (Salihah et al., 2016). Since the post-PCR melting curve analysis further increases the detection time, the protocol in this study used a uniform set of conditions to amplify both *S. aureus* and *B. cereus'* multi-genes multiplex reactions in a single run to reduce the detection time on both bacteria. The use of rapid cycle amplification protocol further reduced the detection time. Hence, this study successfully developed a fast, sensitive, and specific real-time multiplex PCR method to amplify two gene targets for specific detection of *S. aureus* and *B. cereus* with EvaGreen® dye chemistry under a single amplification condition without pre-enrichment step.

# 2 MATERIALS AND METHODS

## 2.1 Genomic DNA of Bacterial Strains

This study used Genomic DNA purchased from American Type Culture Collection (ATCC, Manassas, USA) listed in Table 1, both as reference strains and cross-reactivity analysis**.** The concentration and purity of the genomic DNA was measured by NanoPhotometer™ P-Class (Implen, Munchen, Germany) spectrophotometer by reading off the absorbance at 260 nm and the absorbance $A_{260}/A_{280}$ ratio, respectively. The genomic DNAs were then diluted with 1 × TE buffer to appropriate concentrations before use.

## 2.2 Bacterial Cultivations and Cell Counting

The *S. aureus* ATCC 25923 and *B. cereus* ATCC 14579 live bacterial strains were obtained from Microbiologics Inc (Minnesota, USA). They were cultured in LB broth, Miller (Fisher Scientific, Pittsburgh, USA) at 30 °C for 48 hours. The total cell count of the culture was determined with a Neubauer haemocytometer (Hausser Scientific, Horsham, USA) before inoculating food products with them and their subsequent extractions. The culture broth was concentrated by centrifuging followed by removal of the supernatant broth and addition of 10 mL of 1 × PBS. 1 mL of the cultured broth was heat treated at 100 °C for 10 minutes for safe handling and counting. The heat-treated culture was then serially diluted with 1 × PBS buffer and counted with haemocytometer for at least three times. The non-treated cultured broth was then diluted with 1 × PBS buffer to appropriate concentration before used to inoculate real-food sample.

## 2.3 Oligonucleotides Design and Selections

The oligonucleotides designed and selected for this study are listed on Table 2. The two primer pairs were selected to target *nhe* and *cerA* of *B. cereus,* and *nuc* and *htrA* genes for *S. aureus*. To ensure that the duplex assays for *S .aureus* and *B. cereus* are specific, in-silico analysis with Primer-Blast (National Centre

Table 1: Bacterial strains used in this study.

| Bacteria | Strain no. | Cross-reactivity analysis | | | |
|---|---|---|---|---|---|
| | | *S. aureus* duplex | | *B. cereus* duplex | |
| | | *nuc* | *htrA* | *nhe* | *cerA* |
| *Staphylococcus aureus* | ATCC 25923 | + | + | - | - |
| *Bacillus cereus* | ATCC 14579 | - | - | + | + |
| *Legionella pneumophila* | ATCC 33152 | - | - | - | - |
| *Bacillus subtilis* | ATCC 23857 | - | - | - | - |
| *Salmonella enterica* | ATCC 13311 | - | - | - | - |
| *Escherichia coli* | ATCC 25922 | - | - | - | - |
| *Clostridium perfringens* | ATCC 35401 | - | - | - | - |
| *Shigella flexneri* | ATCC 13124 | - | - | - | - |
| *Campylobacter jejuni* | ATCC 33292 | - | - | - | - |
| *Yersinia enterocolitoca* | ATCC 27739 | - | - | - | - |
| *Aeromonas hydrophila* | ATCC 7966 | - | - | - | - |
| *Plesiomonas shigelloides* | ATCC 51903 | - | - | - | - |
| *Streptococcus pyogens* | ATCC 19615 | - | - | - | - |
| *Cronobacter sakazakii* | ATCC BAA-894 | - | - | - | - |
| *Mycobacterium avium* | ATCC BAA-968 | - | - | - | - |

for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) and OligoAnalyzer Tool (IDT) was carried out.

The suitability of the assays was first analyzed by singleplex real-time PCR. Briefly, the assays were reacted in a 25 µL PCR mixture that contained Ultrapure MilliQ water, 1× of Buffer II, 250 nM of both the forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix (Invitrogen™ Lifetechnologies, Van Allen Way, U.S.A.), 0.1× ROX reference dye (Invitrogen™ Life technologies), 1 × EvaGreen® dye, 0.625U of AmpliTaq DNA polymerase (Applied Biosystem™ Life technologies, Van Allen Way, U.S.A.) and 3 µL of DNA template and were run in duplicates. The amplifications were carried out on the 7500 Fast real-time PCR system (Applied Biosystem™ Lifetechnologies, Van Allen Way, U.S.A.). Fast cycle amplifications were conducted with the singleplex analysis with the initial denaturation at 95° C for 20 seconds, and 40 cycles of denaturation at 95° C for 3 seconds followed by Annealing/extension for 30 seconds at 60 °C.

A step-hold melting curve analysis was also performed after amplifications by heating the PCR mixture at 95 °C for 15 seconds, and then lowering to 60 °C for 1 minute. The temperature was then increased to 95 °C for 30 seconds and the fluorescence signal was monitored at this temperature. The PCR mixtures were then cooled to 60 °C for 15 seconds.

Table 2: List of primer pairs and probes designed and selected.

| Primer name | | Sequence (5'-3') | Product size (bp) | Product GC (%) | Reference |
|---|---|---|---|---|---|
| BCcera | F | TGGAACTGGAAAGGTACG | 200 | 42.5 | This study |
| | R | GTAACACGTTGTGCATCC | | | |
| BCnhe | F | GCATCCAAGAGAGTATGG | 186 | 32.2 | |
| | R | GTTCAGCTTGAATTTCCC | | | |
| SAnuc | F | AATATGGACGTGGCTTAGCG | 196 | 35.7 | Salihah et al., 2019 |
| | R | TGACCTGAATCAGCGTTGTC | | | |
| SAhtra | F | CGTAAGCGTCGTGAATTCTTCC | 208 | 30 | This study |
| | R | CTTCAGCTTTATTCTCATTAACATCACG | | | |

## 2.4 Development of Duplex Real-time PCR Assays

The duplex real time PCR reactions were subsequently reacted in 25 µL of PCR master mix prepared with Ultrapure MilliQ water containing 1× of Buffer II, 100 nM of each primer pairs for *B. cereus* duplex reaction whereas 80 nM and 100 nM for *nuc* and *htrA* primer pairs respectively for *S. aureus* duplex reaction, 4 mM $MgCl_2$, 0.4 mM of dNTP mix (Invitrogen™ Lifetechnologies, Van Allen Way, U.S.A.), 0.1 × ROX reference dye (Invitrogen™ Life technologies), 1 × EvaGreen® dye, 1.25 U of AmpliTaq DNA polymerase (Applied Biosystem™ Life technologies, Van Allen Way, U.S.A.) with 6 µL or 8 µL of DNA template for *S. aureus* and *B. cereus* respectively. The duplex real-time PCR amplifications were performed on the same 7500 Fast real-time PCR system (Applied Biosystem™ Lifetechnologies, Van Allen Way, U.S.A.) in fast cycle amplification. Step-hold melting curve analysis was performed after the amplification, as previously described. All real-time reactions were performed in either duplicates or triplicates.

## 2.5 Qualitative Detection in Milk

A 10-fold serial dilution of *B. cereus* ATCC 14579 and *S. aureus* ATCC 25923 cultures with 1 × PBS

buffer yielded 1 to 1 × $10^3$ cells/µL. For *B. cereus* and *S. aureus* detection in real samples, 200 µL milk samples were artificially contaminated with 1 µL serial dilutions of *B. cereus* ATCC 14579 and *S. aureus* ATCC 25923 cultures DNA was extracted from the milk matrix DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) previously described in Salihah *et al.* (2019). 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: 200 µL of the sample was centrifuged for 30 minutes at 14,000 rpm. The pellet was washed twice with 500 µL of 1 × TE buffer (pH 8.0) before re-suspending in 200 µL of 1 × TE buffer. It was then incubated at 99 ºC for 15 minutes before lysis with 200 µL of AL buffer (containing guanidium chloride, supplied by the kit) and 25 µL Qiagen Proteinase K at 70 ºC for 30 minutes. After heating, 200 µL of 99.8 % ethanol (Sigma-Aldrich, Singapore) was added to the sample and vortexed thoroughly. The mixture was then pipetted into the DNeasy Mini spin column (supplied by the kit) with 2 mL collection tube attached. The column was then centrifuged at 8,000 rpm for 1 minute, collection tube and flow-through were then discarded and replaced with clean new collection tubes (supplied by the kit). Then the column and centrifuge at 8,000 rpm for 1 minute after addition of 500 µL of AW1 buffer (containing ethanol and guanidium chloride, supplied by kit). The liquid in the collection tube was then discarded. Clean and new collection tube was attached to the column and 500 µL AW2 buffer (containing ethanol, provided by the kit) was run through the column followed by a 3-minute centrifugation at 14,000 rpm. Collection tube was discarded and the column was transferred to 1.5 mL autoclaved and UV irradiated microcentrifuge tube. The template was eluted once from the column with 40 µL AE buffer (containing 10 mM Tris-Cl and 0.5 mM EDTA, pH 9, supplied by the kit) whereas, for *S. aureus* DNA extraction, the template was eluted once from the column with 60 µL AE buffer. The column was then incubated at room temperature for 1 minute before centrifugation at 8,000 rpm for 1 minute

# 3 RESULTS AND DISCUSSION

## 3.1 Oligonucleotides Design and Selections

It is necessary to target for than a single gene when using PCR-based methods such as real-time PCR. This is because single gene detections be limiting due to the varying occurrence of genes in different strains of the same bacterial species. For example, the *nhe* and *cerA* genes used for *B. cereus* detection, are present in 65-75% and 90-95% of *B. cereus* strains respectively (De Santis et al., 2008; Martínez-Blanch et al., 2009). While, *nuc* gene that encodes the *S. aureus* specific thermostable nuclease enzyme which has been used to confirm the presence of *S. aureus* in culture-based detection[3] were found in only 75-78% of phenotypically positive *S. aureus* strains in milk and porcine products (Salem-Bekhit et al., 2010; Velasco et al., 2018). While *htrA* gene is consistently found in all *S. aureus* strains (Chiang et al., 2007; Cremonsi et al., 2014), the study on *htrA* gene prevalence in *S. aureus* strains is very limited in comparison to the more commonly used *nuc* gene. Targeting more than a single gene is necessary in comprehensively identifying *S. aureus* and *B. cereus* with real-time PCR.

The primers sequences designed in Table 2 for *S. aureus' nuc* and *htrA* genes and *B. cereus' cerA* and *nhe* genes were analyzed against the sequences in the Genbank database. They were found to be specific to only the target bacteria strains.

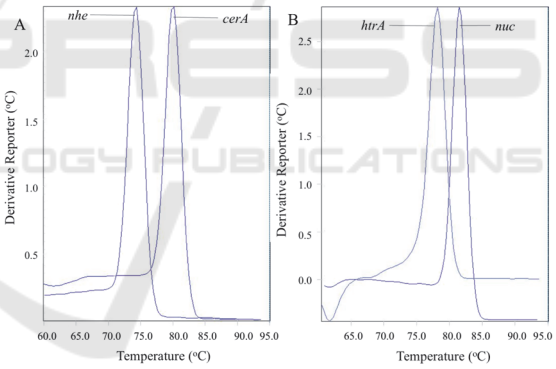


Figure 1: Melting curve analysis of singleplex amplifications of (A) B. cereus' nhe and cerA genes and (B) S. aureus' nuc and htrA genes.

The suitability of the assays were further analyzed in a singplex reactions to ensure that they would produce distinctive $T_m$s in the melting curve analysis for the multiplex reactions. The amplicons (for positive controls) produced single distinguishable melting peaks for both *B.* cereus's *nhe* and *cerA* at 74.2 ± 0.151ºC and 80.0 ± 0.153ºC respectively (Figure 1A) and *S. aureus's htrA* and *nuc* genes at 78.2 ± 0.096ºC and 81.3 ± 0.154ºC respectively (Figure 1B), which proved the suitability of dsDNA binding EvaGreen® dye as the detection chemistry for duplex reactions targeting dual genes of both *B. cereus* and *S. aureus*.

## 3.2 Development of Duplex Real-time PCR Assays

Since, dsDNA binding EvaGreen® dye was used, post-PCR melting curve analysis were performed to ensure that each target in the duplex *S. aureus* and *B. cereus* reactions are distinguishable. Both of the duplex reactions showed that *B. cereus' nhe* ($T_m$ = 78.7 ± 0.136ºC) and *cerA* ($T_m$ = 83.8 ± 0.0783ºC) and *S. aureus' nuc* ($T_m$ = 78.1 ± 0.151ºC) and *htrA* ($T_m$ = 83.8 ± 0.153ºC) amplification produce distinct and easily identifiable melting peaks (Figure 2). Each amplifications produced primer-dimers for the negative controls of both *S. aureus* and *B. cereus* duplex real-time PCR reactions. However, the primer-dimer $T_m$ peaks were lower for both the target genes and were easily differentiated from the target amplicons' $T_m$ peaks.

So they are suitable for the multiplex reaction with the EvaGreen® dye. The variation in $T_m$s of the amplicons are dependent on base compositions and to some extent the length of the amplicons (Nitsche, 2007). This study found that despite the relatively same amplicon lengths of both the targets for *S. aureus* and *B. cereus* duplex detections - the experimented amplicons' $T_m$ were distinctively different. Since the lengths are relatively similar, the guanine and cytosine nucleobases content of the amplicons that contributes more to their $T_m$ difference (Haynie, 2001). As shown in Table 2, the amplicons with higher GC content (*B. cereus'* BCcerA and *S. aureus'* SAnuc) have higher $T_m$ values in comparison to amplicons with lower GC content (*B. cereus'* BCnhe and *S. aureus'* SAhtrA). The reason for this correlation is that nucleobases guanine and cytosine pairs form three hydrogen bonds, which stabilizes the DNA double-helix structure more than the two hydrogen bonds formed by nucleobases adenine and thymine pairs (Marmur and Doty, 1962; Tropp, 2008). Thus, DNA with higher GC content requires more energy to break the triple hydrogen bonds and thus have higher $T_m$ (Marmur and Doty, 1962).

However, the addition of post-PCR melting curve analysis will add to the detection time. Therefore to compensate, the proposed duplex assays (*S. aureus* duplex and *B. cereus* duplex) were specifically design to amplify with the same fast protocol amplification condition, which takes about approximately 30 minutes before post-PCR melting curve analysis. Thus, both duplex assays can run at the same time to reduce the detection time and to streamline the process for detection of both *S. aureus* and *B. cereus* in a single run.

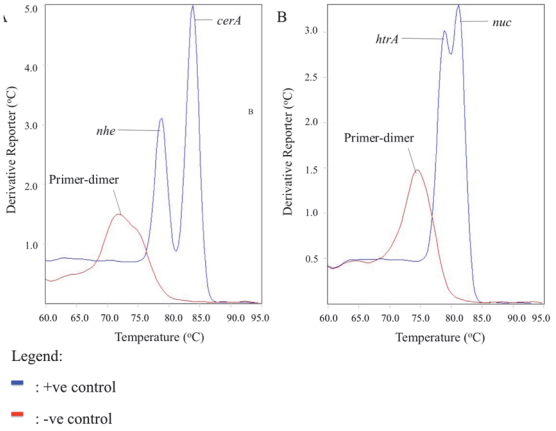


Figure 2: Melting curve analysis for the assay for fast cycle amplification of (A) B.cereus, (B) S. aureus.

## 3.3 Sensitivity and Cross-reactivity Analysis

The sensitivity of the dual targets individual detections of *S. aureus* and *B. cereus* were analyzed for fast amplification protocol. The results of the sensitivity tests are listed in Table 3 for both *B. cereus* and *S. aureus* detection.

Table 3: Sensitivity analysis results for individual duplex detections of B. cereus and S. aureus.

| Bacterial target | Gene | LOD (fg/reaction) | Probability (%) |
|---|---|---|---|
| *B. cereus* | *nhe* | $6.0\times10^1$ | 50.0 |
| | *cerA* | 6.0 | 100.0 |
| *S. aureus* | *nuc* | $3.0\times10^2$ | 83.3 |
| | *htrA* | $3.0\times10^1$ | 50.0 |

The cross-reactivity of the duplex assays for fast cycle amplifications were tested against the other bacterial species listed in Table 1. The post-PCR melting curve analysis did not show any specific $T_m$ peaks for bacterial species other than the peaks for positive controls (*B. cereus* ATCC 14579 and *S. aureus* ATCC 24923). The overall table view of the cross-reactivity results is listed in Table 1 for *B. cereus* and *S. aureus* multiplex reactions.

Therefore, even though combining the fast amplification cycle and multiplexing decrease the sensitivity of the assay, relatively high sensitivities were still obtained for both *S. aureus* and *B. cereus' duplex* assays. The *B. cereus* duplex's limits of detections (LODs) were 1 cell/reaction and 10 cell/reaction for *cerA* and *nhe* genes, respectively. Whereas for *S. aureus* duplex the LODs of 10 cell/reaction and 100 cell/reaction for *nuc* and *htrA* genes, respectively. The sensitivity obtained for both

duplex assays are comparable to the sensitivity obtained for the previous singleplex *B. cereus* and *S. aureus* detection (Salihah et al., 2018; Salihah et al., 2019).

Furthermore, both the duplex assays are highly specific to *B. cereus* and *S. aureus,* free from cross-reactivity with other bacterial species *in-silico* and *in-vitro* (Table 1). Thus both duplex is highly specific and fairly sensitive.

## 3.4 Qualitative Detection in Milk

The suitability of the proposed dual gene targets detection of both *S. aureus* and *B. cereus* was further evaluated with simulated milk samples under the fast cycle amplification condition for simultaneous detection of *S. aureus* and *B. cereus* in a single run. *S. aureus* and *B. cereus* DNAs were directly extracted from milk samples and were then amplified and detected by individual duplex assays in separate PCR tubes and were analyzed together under a single amplification condition. This allowed simultaneous detection of dual gene targets of *S. aureus* and *B. cereus.*

The assay detected *S. aureus* in milk samples having at least 10 cells/reaction while *nuc* gene was targeted while at least 100 cells/reaction was required when *htrA* gene was targeted. The sensitivity obtained for *nuc* and *htrA* gene were comparable to those observed in the sensitivity analysis with pure genomic DNA dilutions (Table 3). Hence, the proposed assay claims the capability of detecting *S. aureus* with as low as 500 cells/mL of simulated milk sample. On the other hand, for *B. cereus* detection sensitivity of 1 cell/reaction was observed for *cerA* gene and 10 cells/reaction for *nhe* gene. This was equivalent to 25 cells of *B. cereus* in 1 mL of artificially inoculated milk.

This indicated the suitability of the assays to detect target bacterial pathogens against background microflora in complex food products such as milk. The suitability were tested practically by using the no pre-enrichment and no-expensive enzymes lysis method of an adapted Qiagen DNeasy blood and tissue kit previously developed by Salihah *et al.* (2019). This further reduced detection time (direct detection without the need of the additional pre-enrichment step) and cost (no need to use expensive enzyme lysis).

# 4 CONCLUSION

Therefore, we developed a real-time PCR dual gene *B. cereus* and *S. aureus* detections system using a single set of amplification condition to run two individual duplex assays with EvaGreen® dye. The assays demonstrated a highly specific and sensitive detection of both gene targets of each species and showed highly specific amplification against a large set of background microflora. Further analysis is needed to assess the applicability of the proposed assay against at least five different strains of *S. aureus* and *B. cereus* to validate both assays according to ISO 16140. Since, analysis of the $T_m$ of the amplicons was a part of the detection method - the reproducibility (i.e. inter- and intra-assay) of the amplicons' $T_m$ needed to be calculated from a range of *B. cereus* and *S. aureus* strains, as shown by Wehrle and colleagues (2010). In addition, it might need to demonstrate the capability of the multiplexed assays in detecting a wide range of strains, and in case of *B. cereus* multiplex assay, an inclusion of other enterotoxigenic *B. cereus* strains might be tested. Furthermore, an inclusion of other target genes for both *B. cereus* and *S. aureus* in the assay might help to find more genetic indicators of the bacterial pathogens. Primer pairs could have been designed to target the *hbl, cytk1* and *ces* genes (Wehrle et al., 2009; Wehrle et al., 2010) to measure the enteropathogenic potential of *B. cereus* strains. For *S. aureus* multiplex detection, the inclusion of primer pairs targeting the enterotoxin gene cluster (*egc*), enterotoxin genes *sea, seb, sec, sed, see, entC* as well as *S. aureus* specific *femA* gene (Tamarapu et al., 2001; Pelisser et al., 2009; Fusco et al., 2011) could be considered.

Overall, the use of dsDNA binding dyes like EvaGreen® dye in this study provides an advantage over probe-based chemistry as it is not only easier to design and cheaper but also free from the limitation of unavailability of compatible probe-dyes for current real-time PCR instruments (Agindotan et al., 2007). In conclusion, the study claims to develop a highly specific and sensitive multiplex assay to detect two target genes of both *B. cereus* and *S. aureus*. This multiplex assay was cost-effective as it used EvaGreen® dyes chemistry and as both multiplex reactions were run under a single amplification condition which gave the benefit of streamlining the detection of *B. cereus* and *S. aureus*.

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