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A novel, rapid, and sensitive real-time PCR assay for cost-effective detection and quantification of *Staphylococcus aureus* in food samples with the ZEN™ double-quenched probe chemistry

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Abstract

Staphylococcus aureus is a prevalent food pathogen. The pathogen is very dangerous as it could survive high cooking temperature and produce heat-stable toxins. Therefore, a fast, sensitive and secure culture-free alternative to detect and correctly quantify *S. aureus* in food samples to prevent food poisoning associated with it is needed. In the present work, the development of a comparatively inexpensive real-time qPCR assay based on the ZEN™ probe chemistry by targeting the thermonuclease gene for a fast, sensitive, cost-effective, and direct quantitative detection of *S. aureus* in real food samples is reported. The novel assay showed high specificity and sensitivity with the limit of detection (LoD) of as low as 1 copy /reaction. The quantification range varies between 1×10^1 and 1×10^7 copies/reaction. This method was able to accurately quantify *S. aureus* from milk sample with a relative accuracy of 75.88 to 120%. The novel assay successfully detected the presence of as low as 50 copies/mL or copies/g of *S. aureus* in real food matrices.

Introduction

Staphylococcus aureus is an opportunistic pathogen and a major causal agent of food poisoning. It contaminates foods by releasing heat-stable enterotoxins which cause a range of self-limiting to life-threatening health conditions in humans (Bannerman, 2003). As low as 10^5 CFU/g of *S. aureus* might produce enough toxins (Bennet and Hait, 2012) to induce gastroenteritis and, in some cases, temporary irregular blood pressure, muscle cramping and headache (Bennet and Monday, 2003). Consumption of *S. aureus*-contaminated food rapidly induces abdominal cramps and pain with nausea, vomiting and diarrhoea. These are, in general, self-limiting with a recovery period between two to three days (Kluytmans *et al*., 1997; Le Loir *et al*., 2003) with reports of severe and fatal cases especially in elderly, infants, young children and severely infirm patients (Le Loir *et al*., 2003).

S. aureus is especially prevalent in raw and unprocessed animal-originated food such as raw meat and poultry, unpasteurised dairy and egg products (Carfora *et al*., 2015). As *S. aureus* could only be destroyed through pasteurisation or high temperature cooking (Puah *et al*., 2016), food poisoning associated with these bacteria typically spread through their introduction into food products that require no additional cooking, e.g. pastries, salad and pudding. This happens through asymptomatic human carriers who have poor hygiene and improperly handle the food (Bennett *et al*., 2013). Moreover, improper handling and storage of food before cooking might cause *S. aureus* food poisoning as the bacteria could grow and produce enterotoxin between 14 and 44°C (Schmitt *et al*., 1990). *S.aureus*' enterotoxin could survive high cooking temperatures (Le Loir *et al*., 2003) thus harming those who ingest the contaminated food even after the bacteria have been killed by the cooking process. Accordingly, it is necessary to not only detect and quantify live bacteria in uncooked food but also to quantify dead bacteria in the cooked food to assess its safety. The latter is, however, not possible to be performed by

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conventional method that requires enumeration of living culturable bacteria (Yousef and Carlstrom, 2003) as this could lead to false negative results. Alternatively, PCR-based methods, utilising genomic DNA of bacteria as the sample to be amplified in a condition of high pressure and temperature and wet environment, could be used for the detection of both dead and viable bacteria (Suyama and Kawaharasaki, 2013; Yap *et al*., 2013). Real-time PCR is a technique based on modification of the PCR technology. It has eliminated most of the post PCR-analyses, and measures the PCR amplification in real-time as the PCR progresses by the inclusion of fluorescence detection chemistry in the mastermix. It could be used for both the detection and the absolute quantifications of bacteria. In addition, advancement in both the real-time PCR instruments (i.e.: rapid cycle or fast real-time PCR system) and the detection chemistry (i.e.: new probe and dsDNA binding dye technology) allow for even faster and more sensitive and accurate detection and quantification of bacteria in food (Salihah *et al*., 2016). Presently, all food-based real-time PCR detection of *S. aureus* involves SYBR Green I or TaqMan probe chemistries. Most methods involve a lengthy pre-enrichment step and a standard real-time PCR cycle (Chiang *et al*., 2007; Cabicarová *et al*., 2016). This is time consuming and does not allow for direct quantification or detection of *S. aureus* in the food. Studies by Hein *et al*. (2001) and Fusco *et al*. (2011) have shown a direct detection of *S. aureus* with rapid cycle real-time PCR with a commercial pre-made mastermix or a more expensive individual component of the mastermix, which overall could be very costly. This is also true with SYBR Green chemistry, considered to be a more cost-effective chemistry in comparison to probe-based chemistries (Salihah *et al*., 2016). To the best of our knowledge, the latter is only available as a pre-made mastermix, which tend to also increase the overall cost of the amplifications. The uses of SYBR Green chemistry, a non-specific dsDNA binding chemistry, also requires an additional post-PCR melting curve analysis which adds an additional time before detection. Furthermore, *S. aureus* is difficult to lyse and generally requires additional lysis with an expensive lysostaphin enzyme in addition to lysozyme, keeping the real-time PCR quantitative detection of *S. aureus* in food cost-prohibitive.

The present work was aimed to develop a sensitive, fast real-time PCR detection and quantification of *S. aureus* directly from milk and other food samples by amplifying the *nuc* gene, while reducing the overall cost. The "rapid" aspect of the method was tackled by using the rapid cycle real-time PCR employing ZEN™ double-quenched probe chemistry (IDT). ZEN™ double-quenched probe is hydrolysis-probe chemistry with an additional ZEN™ internal quencher inserted in between the reporter dye and quencher. This lowers the background signal and theoretically allows for more sensitive and precise detection and quantification and as sequence-specific probe, and like the TaqMan probe, requires no post-PCR melting curve analysis. This further shortens the detection time. Rapid cycle real-time PCR allows for faster detection time compared to standard amplification protocol. The "cost-effectiveness" of the overall method was addressed by using a separate PCR reagent kit that is significantly cheaper and able to substitute the lysis using lysostaphin with a boiling method.

Materials and methods

Genomic DNA of bacterial strains

The Genomic DNA's were obtained from the American Type Culture Collection (ATCC, Manassas, USA), and are listed in Table 1. The concentration and purity of the genomic DNA were measured by NanoPhotometer™ P-Class (Implen, Munchen, Germany) at A_{260} nm and A_{260}/A_{280} ratio, respectively. The genomic DNAs were then diluted to the appropriate concentrations before use with Tris-EDTA buffer (p.H. 8.0).

Table 1. Genomic DNA of bacterial strains obtained from ATCC.

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

Bacterial strains, culture media, growth method and cell counting

The *S. aureus* strain ATCC 25923 was obtained from Microbiologics, Inc (Minnesota, USA). It was cultured in LB broth, Miller (Fisher Scientific,

Pittsburgh, USA) at 30°C for 48 h. The total cell count of the culture was determined with a Neubauer haemocytometer (Hausser Scientific, Horsham, USA), before inoculation and extractions.

Primers and probe design

The primer pairs and probes (Table 2) that target a fragment of the *nuc* gene (Accession number V01281) of the *S. aureus* strain were designed by using the PrimerQuest Tool of Integrated DNA Technologies (IDTDNA, Coralville, USA). Primer-Blast (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) confirmed the exclusiveness of the primer pairs and probes to *S. aureus* strains and OligoAnalyzer Tool (IDT) assured the absence of strong secondary structures (i.e.: primer dimers and hairpin structures) *in silico*. The primer pair and ZEN™ probe, labelled with a fluorescent dye FAM at the 5' end, Iowa black FQ quencher at the 3' end, and additional internal quencher ZEN in the middle of the probe (IDTDNA, Singapore Science Park III, Singapore).

Real-time PCR assay

The assay was carried out on the 7500 Fast real-time PCR system (Applied Biosystems™ Life Technologies, Van Allen Way, USA) in a 25 µL PCR mixture that contained ultrapure water (deionized ~18 MΩ/cm) from Merck Millipore (Burlington, MA, USA), 1× Buffer II, 500 nM both the forward and reverse primers, 250 nM probe, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (Invitrogen™ Life Technologies, Van Allen Way, USA), 0.1× ROX reference dye (Invitrogen™ Life technologies), 0.625 U AmpliTaq DNA polymerase (Applied Biosystem™ Life technologies, Van Allen Way, USA) and 6 µL DNA template and were run in triplicate. ROX is a reference dye required by the ABI 7500 Fast real-time PCR system to normalise tube-to-tube signal difference.

Rapid cycle amplification was conducted with the following steps: initial denaturation at 95°C for 20 s, 40 cycles of denaturation at 95°C for 3 s, and annealing/extension for 30 s at 60°C. Millipore water was used as negative control throughout the experiment.

Real-time PCR assay specificity

The inclusivity and exclusivity of the primer pairs and probes were assessed *in silico* by using Primer-Blast (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), and experimentally with real-time PCR assays as described above with 3×106 fg of genomic DNA of bacterial strains as listed in Table 1.

Detection limit and probability

The detection limits of both assays were determined experimentally against serial dilutions of genomic DNA *S. aureus* ATCC 25923, and were repeated three times for probability calculations.

Assay performance

The DNA standard curve was constructed by using 10-fold serial dilutions of the genomic DNA of *S. aureus* ATCC 25923 to create concentrations ranging from 1 copy number to 1×10^7 copy number/reaction to analyse the assay performance and quantitative capabilities of both assays. The copy number of the *nuc* gene was determined based on the size of the *S. aureus* whole genome of 2,778,854 bp (Treangen *et al*., 2014). As the *nuc* gene is a single copy gene, one copy number of *nuc* gene was equivalent to 3 fg of the genomic DNA of *S. aureus.*

Artificial contamination of food samples

Sterile skimmed milk was inoculated with serial dilutions of *S. aureus* ATCC 25923 from 50 to 5×10^7 copy no/mL of *S. aureus* in milk to confirm the detection of the bacteria by the proposed method and to determine the relative accuracy of the quantification. The applicability of the protocol to a wider assortment of foods was tested by determining the limit of detection of *S. aureus* in other food samples including canned tuna, canned corned beef, liquid egg, cooked sausages, custard pastry, cream pudding, chicken curry bun, potato salad (previously tested negative for *S. aureus* with real-time PCR). The food samples were inoculated with known dilutions of *S. aureus* ATCC 25923 followed by extraction of the bacterial DNA and analysis using both *SaNucP1* and *SaNucP2* ZEN™ probe assays. The bacterial DNA was extracted from the 200 µL milk matrix and 200 mg homogenised food samples by using the adapted DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany. Solid food samples were homogenised with mortar and pestle within a sterile plastic bag. The protocol was modified as follows: 200 µL or mg sample was centrifuged for 30 min at 21,000 g. The pellet was washed twice with 500 µL 1× TE buffer (pH 8.0), re-suspended in 200 µL 1× TE buffer, incubated at 99°C for 15 min and centrifuged for 15 min at 21,000 g. The supernatant was then lysed with 200 µL AL and 25 µL Qiagen Proteinase K at 70°C for 30 min. Following heating, the rest of the protocol from DNA extraction of Gram-positive bacteria up to the DNA elution step was performed as per manufacturer's instructions. DNA was eluted at once from the column with 60 µL AE buffer. For real-time PCR analysis, 6 µL extracted DNA samples was used as template.

Results

Primer and probe design and specificity

The sequences of the primer pairs and probe were designed with PrimerQuest Tool software to target the *nuc* gene (Table 2), and were found in the GenBank database to be homologous to only *S. aureus* strains. This corroborated with the cross-reactivity analysis that showed specificity of both the assays to target strain against other non-*S. aureus* bacteria strains as listed in Table 1.

Detection limit and probability

The developed *SaNucP1* and *SaNucP2* ZEN™ probe assays (Table 2) were highly sensitive and detected as low as 1 copy number/reaction or 3 fg/reaction, with 33.33% and 22.22% probability respectively. LoD indicated the highest dilutions where observable positive amplification could be obtained in the replicates (Martínez-Blanch *et al*., 2009).

Assay performance

The performance of the ZEN™ double-quenched probe assays designed in Table 2 was analysed by constructing the standard curves and determining the efficiency, R^2 value, with inter- and intra- coefficient variations of each assay under the fast, real-time PCR protocol. The standard curves were generated from 10-fold dilutions of *S. aureus* ATCC 25923 genomic DNA in the range of 1 to $1x10^7$ copy number/reaction (Table 3).

The efficiency was calculated from the standard curves based on the equation described by Klein *et al*. (1999). The *SaNucP1* assays showed 97.17% efficiency (R^2 = 0.999) as compared to 94.97% (R^2 = 0.999) of the *SaNucP2* assays. Both were within the recommended efficiency (90 to 110%) and R^2 > 0.99 for real-time PCR (Johnson *et al*., 2013). Both *SaNucP1* and *SaNucP2* probe assays showed working range between 1 to 1×10^7 copy number/reaction. The *SaNucP1* probe assay showed higher linear quantification range (Table 3) of 1×10^1 to 1×10^7

Table 2. The designed probes and primers for the quantitative analysis of *S. aureus*.

Assay		Sequence (5'-3')	Locations	Size (bp)
SaNucP1	F[a]:	AGTGGTTCTGAAGATCCAACAG	433-632	192
	P[b]:	6FAM-TGGTTGATA(ZEN)CACCTGAAACAAAGCATCCT-1BFQ		
	R[c]:	CAGGACCATATTTCTCTACACCTT		
SaNucP2	F[a]:	AATATGGACGTGGCTTAGCG	713-909	196
	P[b]:	6FAM-AGCTTTAGT(ZEN)TCGTCAAGGCTTGGCT-IBFQ		
	R[c]:	TGACTCGTCTGAATCAGCGT		

[a]forward primer sequence, [b]probe sequence, [c]reverse primer sequence

Table 3. Ratio of positive reaction and inter- and intra-assay coefficient variations (CV%) for the *SaNucP1* and *SaNucP2* qPCR assays within the range of 1 to 1×10^7 copy numbers of *S. aureus* DNA dilutions using the ZEN™ double-quenched probes chemistry.

Assay	Cell/reaction	Ratio of positive reactions[a]	Efficiency (%)	R^2	Mean CV% ± SD[b]	
					Intra-assay	Inter-assay
SaNucP1	1×10^7	9/9	97.170	0.999	0.504 ± 0.068	0.504 ± 0.123
	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	9/9				
	1	3/9				
SaNucP2	1×10^7	9/9	94.970	0.999	0.486 ± 0.073	0.486 ± 0.173
	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	9/9				
	1	3/9				

[a]number of positive result per nine individual reactions, [b]SD = standard deviations

copy number/ reaction with limit of quantification (LoQ) at 1×10^1 copy number/reaction. LoQ refers to the highest dilutions with amplifications at >95% probability (Fusco *et al*., 2011). In comparison, dynamic linear quantification range of the *SaNucP2* probe assay was lower by 1 log unit and ranged between 1×10^2 and 1×10^7 copy number/reaction with LoQ of 1×10^2 copy number/reaction. Both the assays were highly reproducible with approximately less than 1% mean intra- and inter-assay variations (CV%) (Table 3). In summary, both the assays were suitable for the quantification of *S. aureus* with high efficiency, R^2 and quantification range.

Quantification and detection in inoculated food samples

The suitability of the developed novel primer pairs and ZEN™ probes in detecting and quantifying bacteria directly in food samples were further evaluated with skimmed milk samples that were artificially contaminated with serial dilutions of *S. aureus* ATCC 25923 cells ranging from 1 copy number/reaction to 1×10^6 copy number/reaction. 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 required no additional expensive enzymatic lysis (Hossain Ripon *et al*., 2011).

The *SaNucP1* ZEN™ probe assay successfully detected as low as 1 copy number/reaction of real-time PCR assay or 50 copy numbers of *S. aureus* in 1 mL inoculated skimmed milk without any pre-enrichment. The accuracy of the quantification in inoculated milk was evaluated by comparing the values extrapolated from the standard curve that was generated from the DNA dilution standards (Table 4). The relative accuracy of the extraction method in combination with the *SaNucP1* ZEN™ probe assay was between 74.145% to 115.342%, for the linear quantification range between 1×10^1 to 1×10^6 copy number/reaction i.e. 500 to 5×10^7 copy number/mL *S. aureus* in skimmed milk. The *SaNucP1* assay also achieved the same sensitivity of 50 copy number/g of *S. aureus* in other food matrices tested (i.e. tuna, liquid egg, sausages, custard pastry, cream pudding, chicken curry bun, and potato salad) except for *S. aureus* in corned beef that showed a higher LoD of 100 copy number/g. Thus, the working range of *SaNucP1* assay in milk and other food samples was 50 to 5×10^7 copy number/mL or g while a slightly higher working range of between 100 to 5×10^7 copy number/g was used in corned beef.

In comparison, the *SaNucP2* ZEN™ probe assay showed a lower sensitivity as it detected 2 copy number/reaction or 100 copy number/mL *S. aureus* in artificially contaminated skimmed milk with relative accuracy of between 74.004% to 110.617% for linear quantification range between 10^2 to 1×10^6 copy number/reaction i.e. 5×10^2 to 5×10^7 copy number/mL. The same sensitivity was obtained for other food samples specifically corned beef, potato salad and chicken curry bun with LoD as low as 100 copy number/g. Thus, the working range of *SaNucP2* assay for the aforementioned food samples was between 100 to 5×10^7 copy number/mL or g. However, a higher sensitivity was observed for other food matrices including tuna, liquid egg, custard pastry, pudding cream and sausages, with sensitivity down to 50 copy number/mL or g with working range of 50 copy numbers to 5×10^7 copy number/mL or g.

Discussion

Conventionally, culture-based method has been used to detect S. aureus. However, Ikeda *et al*. (2005a), showed the unsuitability of culture-based in quantitative detection of *S. aureus* in implicated food that had already been heat-treated since heat treatment

Table 4 Quantitative detection of *S. aureus* in artificially inoculated milk using *SaNucP1* and *SaNucP2* ZEN™ probe assays.

Cell/ reaction[a]	Ratio of positive reactions[b]	*SaNucP1* Estimated no. ± SD[c]	Relative accuracy (%)	Ratio of positive reactions[b]	*SaNucP2* Estimated no. ± SD[c]	Relative accuracy (%)
1×10^6	3/3	$7.415\times10^5 \pm 6.211\times10^4$	74.145	3/3	$7.400\times10^5 \pm 3.046\times10^4$	74.004
1×10^5	3/3	$1.153\times10^5 \pm 8.622\times10^3$	115.342	3/3	$1.106\times10^5 \pm 1.230\times10^4$	110.617
1×10^4	3/3	$1.015\times10^4 \pm 461.33$	101.517	3/3	$1.029\times10^4 \pm 783.33$	102.85
1×10^3	3/3	$9.027\times10^2 \pm 6$	90.267	3/3	$9.89\times10^2 \pm 59.333$	98.9
1×10^2	3/3	91.667 ± 6	91.667	3/3	$1\times10^2 \pm 14.33$	100.00
10	3/3	10 ± 1	100.00	2/3	d	d
1	2/3	d	d	0/3	e	e

[a]Approximate quantity of genomic DNA (fg/reaction) of *S. aureus* ATCC 25923, [b]Number of positive results per reactions, [c]Estimated quatity of *S. aureus* in milk (fg/reaction) based on DNA dilution standard curve, [d]Values below limit of quantification, [e]No amplification observed

killed the bacteria. The heat-stable enterotoxins remained and poisoned the food as shown by results from the culture-based method (Ikeda *et al*., 2005b). This necessitates the need to develop a method that could quantitatively detect inactivated and activated bacteria. It could serve as an indication of the safety of food, where a minimum of 10^5 CFU/g of *S. aureus* in food is enough to produce enterotoxin to induce food-poisoning. Real-time PCR is suitable to quantitatively detect the DNA of all bacteria present in the food (Ikeda *et al*., 2005a) as it detects the amount of genomic DNA of the bacteria, regardless of its viability, allowing for more accurate measurement of its safety especially for heat-treated food.

In the present work, the *nuc* gene was targeted for oligonucleotide design. The gene encodes for thermonuclease, a heat stable protein that hydrolyses the DNA and RNA. The presence of *S. aureus* could be confirmed by thermonuclease production in culture (Bennett and Lancette, 2001). Thus, novel primers and probes (Table 2) were designed to target the region 433 bp to 632 bp and 713 bp to 909 bp of the *nuc* gene of *S. aureus* (Accession number V01281). It should, however, be noted that other Staphylococcus species other than *S. aureus* also show thermonuclease activity (Gudding, 1983) for which Chiang *et al*. (2007) argued that the *nuc* gene specific primer pairs and probes might produce cross-reactivity with other Staphylococcal isolates other than S. aureus. However, compared to other Staphylococcus species, *S. aureus* produced an identifiable and specific thermostable nuclease enzyme (Brakstad and Maeland, 1989). Thus, the *nuc* gene that encodes the thermostable nuclease enzyme, is also S. aureus-specific. Preliminary results of both *in silico* and *in vitro* analyses had confirmed the specificity and inclusiveness of the designed primer pairs and probes to the *S. aureus* strains. Combining these confirmations with the fact that *nuc* is a single copy gene, it was concluded that specific and direct quantification of *S. aureus* could be attained based on the number of the *nuc* gene present in the samples.

As previously mentioned, the aim of the present work was to develop a fast, sensitive, and cost-effective real-time PCR-based method for the quantitative detection of *S. aureus* in food. Thus, rapid cycle or fast real-time PCR protocol with sequence-specific ZEN™ double-quenched probe chemistry were implemented to reduce the reaction time further from approximately 2 h to 30 min without the need for post-PCR melting curve analysis. However, both the rapid cycle and probe chemistry might affect the sensitivity of the assay since the sensitivity is typically traded-off for probe chemistry specificity and the "rapidity" of the rapid cycle (Hilscher *et al*., 2005; Josefsen *et al*., 2012). However, for the novel primer pairs and ZEN™ probe assays in the present work, a high sensitivity of one copy number/reaction was still achieved under the fast cycling protocol and the probe chemistry used. This was 6× lower than a previous study targeting the *nuc* gene with rapid cycles (Hein *et al*., 2001), and has a comparative sensitivity with other real-time PCR assays targeting other gene targets using a standard cycling protocol (Chiang *et al*., 2007; Cabicarová *et al*., 2016) and rapid cycle protocol (Fucso *et al*., 2011). Despite using the rapid cycle and the probe-based chemistry, a high sensitivity was still obtained.

To further reduce the overall detection time, direct detection from food was also performed to avoid long pre-enrichment time of between 10 to 24 h (Chiang *et al*., 2007; Cabicarová *et al*., 2016). The target bacteria were cultured using enriching broth for the pre-enrichment before DNA extractions. While pre-enrichment might significantly increase sensitivity down to 1 copy number/g of food (Cabicarová *et al*., 2016) (Table 5), it takes a long time and might not work with heat-treated food (Ikeda *et al*., 2005a). In addition, it might not accurately quantify the initial amount of *S. aureus* present in food samples since the pre-enrichment with real-time PCR method would quantify the amount of *S. aureus* in the enriched sample and does not directly indicate the initial amount of *S. aureus* in the sample.

The present work used Qiagen DNeasy blood and tissue kit which had previously been shown (Giacomazzi *et al*., 2005; Elizaquível and Aznar, 2008; Lusk *et al*., 2013) to produce a high quality and quantity bacterial DNA from food. The Qiagen DNeasy blood and tissue kit was adapted by combining it with the boiling step at 99°C to replace the expensive lysostaphin and lysozyme required to lyse *S. aureus* cells thus reducing the overall cost compared to existing assays (Table 5).

The present work showed that the proposed method in combination with the developed *SaNucP1* ZEN™ probe assays could detect as low as 50 copy number/mL or g which was lower than previous studies without the pre-enrichment step (Hein *et al*., 2001; Chiang *et al*., 2007; Fusco *et al*., 2011; Cabicarová *et al*., 2016) with eight different food matrixes (Table 5). However, a higher detection limit was obtained for corned beef sample (100 copy number/g). This could be attributed to its high fat content - a known PCR inhibitor (Schrader *et al*., 2012) and therefore could interfere with the amplification process and lower the sensitivity. Thus, to lower the LoD of *S. aureus* in food with high fat content such as corned beef, further analysis comparing other commercial extraction kits might be necessary especially those

Table 5. Comparison of amplification run times and costs, and extraction costs of the present work and other published studies.

Paper	Chemistry	Reaction time[a]	Pre-enrichment	Real-sample sensitivity	Amplification cost per reactionb (USD)	Extraction cost per reaction (USD)
The present work	Zen Probe	22 in 20 s	no	50 copy no./g or mL	0.355	3.260
Hein *et al*. 2001	TaqMan probe	48 min 35 s	no	75 copy no./g	1.567	8.790
Cabicarová *et al*., 2016	TaqMan probe	71 min 15 s	no	10^3-10^5 copy no./g	0.777	5.286
			24 h	1 copy no./g		
Chiang *et al*., 2007	SYBR Green	70 m 20 s	no	1 to 9 × 10^3 copy no./g or mL	2.075	6.060
			10 h	1 to 9 copy no./g or mL		
Fucso *et al*., 2011	TaqMan probe	22 min 20 s	no	100 copy no./mL	1.321	3.341
	SYBR Green	102 min 20 s		100 copy no./mL	1.02	

[a]Approximate PCR amplification time based on PCR amplification (i.e.: initial denaturation, denaturation, annealing/extension and number of cycles) and melting curve analysis protocols (i.e.: time taken for heating and cooling) if applicable.
[b]Approximate minimum cost calculated based on online price of products' smallest available unit. Calculation exclude the price of any similar reagents used (i.e.: primers) and reagents used without stated origins (i.e.: TE buffer, $MgCl_2$, EDTA, etc.).

specifically developed for DNA extraction from food. The sensitivity could be further increased by increasing the amount of sample extracted and combining this with a filtration system to filter out the bacteria from the food samples and then extracted as described above. The developed assay also produced a wide quantification range in milk between 50 to 5×10^7 copy number/mL in skimmed milk (i.e.: 1×10^1 to 1×10^6 copy number/reaction), which is in line with the linear quantification range obtained with pure genomic DNA (1×10^1 to 1×10^7 copy number/reaction). The relative accuracy of quantification was also close to 100% as shown in Table 4, thus highly accurate in quantifying *S. aureus* in milk.

The *SaNucP2* assay meanwhile has also shown a similar high sensitivity of 50 copy number/mL or g of some food matrixes (i.e.: tuna, liquid egg, custard pastry, cream pudding, sausages). However, it had a higher LoD of 100 copy number/mL or g for the other tested food samples such as milk, corned beef, potato salad and chicken curry bun. The *SaNucP2* assay was more susceptible to PCR inhibitors in some food products and produced a lower sensitivity in comparison to *SaNucP1* assay for similar sample. The assay also had comparatively lower linear quantification range by 1 log unit. Thus, *SaNucP1* ZEN™ probe assay comparatively provided a more sensitive and wider linear quantification range compared to the *SaNucP2* assay.

We had further reduced the cost to compensate the use of the comparatively expensive ZEN™ probe assay and had used significantly economical separate component of a PCR reagent kit added with ROX reference dye rather than using a more expensive commercial pre-mix real-time PCR mastermix (Chiang *et al*., 2007; Fusco *et al*., 2011) or the more expensive PCR reagent kit (Hein *et al*., 2001; Cabicarová *et al*., 2016). Thus, in comparison to published assays for direct detection of *S. aureus* in food sample (Table 5), the assay developed in the present work was comparatively 'cheaper' than SYBR Green I dye chemistry-based assays (Chiang *et al*., 2007; Fusco *et al*., 2011) and the TaqMan-based assays (Hein *et al*., 2001; Fusco *et al*., 2011; Cabicarová *et al*., 2016).

Conclusion

In conclusion, the present work had developed a highly sensitive and fast real-time PCR assay for rapid, more cost-effective, and direct, quantitative detection of *S. aureus* by the *nuc* gene-based ZEN™ probe. The method did not require pre-enrichment, and directly detected and reliably quantified *S. aureus* in artificially inoculated skimmed milk. The adaptability of the proposed probe system to rapidly detect very low concentrations of *S. aureus* in other food matrices without the need of pre-enrichment was also demonstrated.

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