

Comparison of quantitative real-time PCR targeting *nuc* gene and culture-based plate count methods for quantification of *Staphylococcus aureus* in raw cow milk

Enquebahe K. Tarekgne¹

¹Mekelle University College of Veterinary Sciences, P.O. Box 1118, Mekelle, Tigray, Ethiopia.

Corresponding author: Tel. +251-9-14-72-3692, e-mail: enquebahe@gmail.com, Enquebahe.kas-saye@mu.edu.et

Abstract

Staphylococcal food poisoning (SFP) is caused by ingestion of enterotoxins produced by enterotoxigenic *Staphylococcus aureus* when the cell population exceeds 5 Log CFU per gram/ml of food. The Objectives of this study were to evaluate the performance of SYBR Green 1-based quantitative real-time PCR (qPCR) targeting the *nuc* gene for the quantification of *S. aureus* in milk and to compare the assay with the plate count method. The qPCR and the plate count were applied for the quantification of *S. aureus* in 92 naturally contaminated and artificially spiked bulk milk samples. Optimized standard curves were generated as the qPCR employed the absolute quantification method. The qPCR assay discriminates *S. aureus* from other *Staphylococcus* species with a large difference in quantification cycle (C_q) (Mean *S. aureus* C_q = 13.83 ± 0.93; other staphylococci C_q = 30.34 ± 2.65). The standard curve showed 91 % amplification efficiency and 0.98 coefficients of correlation (R²). The detection and quantification limit of the assay was 18 copies of the *nuc* gene. The precision of the assay as expressed by standard deviation was 0.12 – 0.3 for intra-assay and 0.29 – 0.5 for inter-assay variability. In artificially contaminated milk, the R² between CFU ml⁻¹ and *S. aureus* cell equivalent (SCE) ml⁻¹ was 0.95, which implies, the estimation of CFU ml⁻¹ in raw milk by qPCR is possible. A statistically significant (p < 0.05) difference in *S. aureus* count was documented between qPCR and plate count. The average SCE (5.59 ± 1.55 Log SCE ml⁻¹) estimated by qPCR was one Log higher than CFU (4.46 ± 1.06 Log CFU ml⁻¹) estimated by plate count. Furthermore, 28% of the samples with < 5 Log ml⁻¹ *S. aureus* by plate count had > 5 Log ml⁻¹ by qPCR. Hence, the qPCR is recommended for routine and research work for its advantage of rapid, sensitivity, and reliability. Further study on validation of the qPCR protocol in different food matrixes for quantification of foodborne pathogens and cost-benefit analysis of the assay is required.

Keywords: *S. aureus*; qPCR; nuc gene; bulk milk.

Introduction

Staphylococcal food poisoning (SFP) is an important foodborne disease worldwide. *S. aureus* has many potential virulence factors; staphylococcus enterotoxin (SE) is one among others which is responsible for food poisoning. Ingestion of less than 1.0 µg of SE causes SFP (Cretenet, 2011). Milk and milk products are commonly associated with SFP. Human and mastitis cows are the major sources of *S. aureus* to contaminate foods including milk and milk products (Hennekinne *et al.*, 2011; Loir *et al.*, 2003). *S. aureus* has been reported at a prevalence of 38.7% from cow bulk milk and milk products in the Tigray region (Tarekgne *et al.*, 2015).

Quantitative data of *S. aureus*, if any, in raw milk and dairy products during production, processing, and storage is of food safety concern. A high *S. aureus* counts in the product is an indicator of poor hygiene and the possibility of SE production. Enterotoxigenic *S. aureus* can produce SE at levels that can cause illness when the population exceeds 10⁵ CFU per gram of contaminated food (Food and Drug Administration, 2012). However, a low count of *S. aureus* in food does not necessarily indicate the absence of SE. Although large bacterial populations, which are the source of SE, may die during the process, the SE can still survive in products, as it is thermostable and resistant to different enzymatic actions (Hennekinne *et al.*, 2011; Loir *et al.*, 2003).

Several countries set a microbiological criterion for the presence of *S. aureus* in milk and dairy products to be taken as microbiological standards. For example, the European Commission, directive 92/46/EEC, stated that in raw milk intended for processing, < 500 CFU/ml of *S. aureus* is considered an acceptable level. The enumeration of *S. aureus* is not only important as a microbiological criterion but also is a prerequisite for the microbial risk assessment study of SFP (Postollec *et al.*, 2011). In the diagnosis of SFP outbreak enumeration, 10⁵ CFU/g of *S. aureus* in the food remnants is confirmatory (Hennekinne *et al.*, 2011).

There are two methods for the detection and enumeration of *S. aureus* in milk and milk products; the culture-dependent (conventional) and the culture-in-

dependent (molecular) methods. The conventional culture-dependent methods are laborious and time-consuming (Alarco'n *et al.*, 2006).

Real-time quantitative PCR (qPCR) is one of the culture-independent methods that detects and quantifies *S. aureus* and other pathogens from different foodstuffs. Compared to the culture-based methods, qPCR is fast, sensitive, and more specific (Postollec *et al.*, 2011). It enables quantifying specific nucleic acids in a complex mixture even if the initial amount is very low. The quantification of the DNA is performed by monitoring the amplification of the target sequence in *real-time* using fluorescent technology. Although many detection chemistries are available, in food microbiology, SYBR Green I and the Taqman or Molecular Beacons are common (Fraga *et al.*, 2014).

For the detection and enumeration of *S. aureus* many molecular markers have been developed and tested such as the enterotoxin genes (Johnson *et al.*, 1991), 23S rDNA (Straub *et al.*, 1999), and *femA*, *fmhA* genes (Riyaz-Ul-Hassan *et al.*, 2008). The *nuc* gene was initially developed by (Brakstad *et al.*, 1992) and was widely used as a molecular marker for the detection and enumeration of *S. aureus* from clinical and food samples. However, it has been reported to form a primer dimer and non-specific products when used with SYBER Green I in qPCR assay (Hein *et al.*, 2001b). To solve this problem, a new SYBER Green-based primer that targets the *nuc* gene has been developed, tested, and reported as efficient (Alarco'n *et al.*, 2006). As SYBER Green I is cheaper than the TaqMan probe and the designed primers are appropriate, it is important to evaluate and assess qPCR based on these items for local use. If one intended to introduce the qPCR for local use as an alternative approach to the conventional culture-based method, it is imperative to assess and evaluate the qPCR assay in the local food matrixes and local pathogen strains.

Therefore, the objectives of this study were to evaluate the qPCR that targets the *nuc* gene and uses SYBER Green I as a dye for quantification of *S. aureus* in raw cow's milk and to compare the assay with the conventional plate count method.

Materials and methods

Microbiological analysis

A total of 310 samples (168 raw bulk milk and 142 milk products) were collected from the Tigray region, Northern part of Ethiopia with the objective of detection and enumeration of *S. aureus*.

The isolation and enumeration of *S. aureus* from the samples were performed as detailed by (Ahmed, 2003a). Briefly, from the raw milk samples tenfold serial dilutions were prepared, and from each dilution step 100 μ l were inoculated onto duplicate plates of Baird-Parker agar (Oxoid, England) supplemented with egg yolk tellurite (Merck, Germany). The plates were incubated aerobically at 37 °C for 48 hours. Typical colonies, which were circular, black to gray-black, and surrounded by an opaque halo clear zone, were counted. Gram staining was made for each colony and tested for catalase activity. Five typical colonies were subcultured onto brain heart infusion (BHI) (Oxoid, England) and incubated overnight at 37 °C. From the BHI broth cultures, a coagulase test on rabbit plasma with EDTA (Remel, USA), DNase test (Sigma-Aldrich, USA), and anaerobic fermentation of mannitol (Sigma-Aldrich, USA) was performed.

Final identification of the isolates to species level was done by sequencing the 16S r RNA gene.

DNA Extraction: DNA was extracted from the overnight BHI growth by Gen-Elute™ bacterial genomic DNA kit (Sigma- Aldrich, USA) as per the manufacturer's guideline. In brief, one ml of the broth was pelleted by 16, 363 x g for 2 min and washed twice with 0.9% aqueous NaCl solution at the same speed. The content was homogenized with 200 μ l of lysosome solution and incubated at 37 °C for 90 minutes. The suspension was treated with 20 μ l of RNAase and Proteinase K followed by 200 μ l of lysis solution C and incubated at 55 °C for 60 min. The lysate was homogenized with 200 μ l of ethanol to precipitate the DNA and transferred into a new binding column where it was centrifuged at 3968 x g for 1 min. The content was washed twice with washing solution I. Then, the concentrated wash solution and the elute were discarded. Finally, the column was put into a new 2 ml tube and the DNA was harvested with 100 μ l (twice 50 μ l each) elution buffer solution. The concentration (ng/ μ l) and quality (260/280 and 260/230 ratio) of the DNA was determined by NanoDrop

ND-2000 spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). The genomic DNA was stored at -20°C until use for the next 16srRNA gene sequencing and later for qPCR assay.

The forward and reverse universal primers described by Vebo *et al.* (2011) were used to amplify the 16 S rRNA gene with the expected 1200 bp product. The amplification was conducted with a final PCR reaction volume of 50 μl . It contained, 5 μl of 10x ThermopolTm reaction buffer (BioLabs, New England, USA), 0.25 μl Taq Polymerase of 5,000 $\mu\text{g}/\text{ml}$ (BioLabs), 1 μl of each primer of 10 pMol con, 1 μl of 10 Mm deoxynucleotide triphosphate mixture (Sigma-Aldrich, USA) 2 μl of genomic DNA and finally adjusted to a final volume of 50 μl with Milli-Q water. The amplification was carried out in a C1000 TMTthermal cycler (BIO-RAD laboratories) programmed to initial denaturation of 95°C for 1 min, 30 cycles of 95°C for 30 sec., 55°C for 30 sec. 68°C for 80 s and a final extension period at 68°C for 5 min. After amplification, the products were verified by gel-electrophoresis, purified, and sent to GATC Biotech AG (European Genome and Diagnostic Centre, Germany) for sequencing as per the company's instruction. The sequence result was bio-edited and compared to GenBank using the nucleotide BLAST algorithm (<http://www.ncbi.nlm.gov/blast>).

Among the total 120 milk and milk product samples positive for *S. aureus*, 92 were randomly selected for qPCR assay.

The qPCR assay

Bacterial isolates and strains used in the study: The *S. aureus* strain MSSA 476 (accession no. BX571857) purchased from National Veterinary Institute, Debre-Zeit, Ethiopia, was used as a reference strain for the study. It was used for specificity study, artificial contamination of milk, preparation of standard curve, and in all cases as a positive control.

Specificity study

For the specificity study, the following confirmed twelve *staphylococcus* species, namely *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus warneri*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus carnosus*, *Staphylococcus scineri*, *Staphylococcus hominis*, *Staphylococcus divresia*, *Staphylococcus chromogenes*, *Staphylococcus saprophyticus*, and *Staphylococcus caprie*, which were previously isolated from dairy products of

the Tigray region were used (Tarekgne *et al.*, 2015). DNA was extracted from overnight cultures of reference *S. aureus* and other 12 species of *Staphylococcus* in BHI using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instruction, as described previously. To assess the specificity of the primers, the DNA of the isolates involved in the specificity study (n=12), were standardized to 5 ng/μl concentration and subjected to qPCR assay using the same PCR equipment and reaction conditions in duplex. MillQwater was used as a negative control. Then mean Cq and TM values with standard deviation were calculated.

Artificial contamination of raw milk

Raw milk from a healthy cow was collected and confirmed to be free from *S. aureus* by conventional microbiological as well as molecular methods to check for any *S. aureus* DNA. The milk was artificially contaminated by the reference *S. aureus* strain and subjected to molecular analysis as per the method described by (Hein *et al.*, 2005). Briefly, the reference *S. aureus* strain was cultured in BHI broth (Oxoid, England) at 37 °C for 15 hours. From this broth, five tenfold dilutions, from 10⁻¹ to 10⁻⁵, (1 ml of broth to 9 ml peptone water) were prepared with peptone water (Sigma-Aldrich, Switzerland). From each dilution, 100 μl was inoculated in duplex onto Baird-Parker agar (Oxoid, England) supplement with egg yolk tellurite (Merck, Germany) incubated at 37 °C for 48 hours, and CFU/ml of each dilution was calculated as per Ahmed (2003b). Furthermore, from each of the above dilutions, (10⁻¹ to 10⁻⁵), 100 μl was added, to five, 900 μl of raw milk to obtain a tenfold dilution of 10⁻¹ to 10⁻⁵ bacteria spiked raw milk. The artificially contaminated raw milk samples were subjected to DNA extraction, as described below, in triplicate and the *nuc* gene copy number was measured twice by qPCR.

DNA extraction from Milk samples

Ninety-two milk samples (n=92) and the five tenfold dilution (10⁻¹ to 10⁻⁵) of the artificially contaminated milk in triplicate (n=15), were subjected to DNA extraction as follows. Four ml of the content was added to 6 ml of sterile 2% w/v sodium citrate in 15 ml Eppendorf tube and centrifuged for 5 minutes at 16 x g rpm in 4 °C. As much as possible the fat layer was removed with sterile swab tips and the supernatant was transferred into a new 10 ml Eppendorf tube and pelleted at 4500 x g for 15 minutes in 4 °C and transferred into 1.5 ml Eppendorf tube. The content was washed twice with 1 ml of 2% w/v sodium

citrate solution by centrifuging at 16,363 x g for 2 min. in 4 °C and subjected to DNA extraction with GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as described previously.

Quantification Standard for qPCR

The reference *S. aureus* was cultured in BHI broth (Oxoid, England) at 37 °C overnight. One ml of the broth (pure culture) was subjected to DNA extraction as per the aforementioned procedure. After quantification of DNA concentration by Qubit® 2.0 Fluorometer assay (Invitrogen, USA), tenfold dilutions containing 1.8×10^1 to 1.8×10^5 copies of *nuc* gene were prepared assuming that 1 ng of DNA equals 6×10^5 times the entire genome and that the *nuc* gene is a single-copy gene (Hein *et al.*, 2001a), hence 0.003 ng/μg equals to 1.8×10^2 *nuc* gene. Each dilution was subjected to qPCR assay in triplicate. For optimization purposes, many runs have been conducted till the best standard curve is acquired.

The slope(s) of the standard curve was used for the calculation of the PCR efficiency using the equation $E = 10^{-1/s} - 1$ Where E=efficiency, and s=slope. The qPCR assay employed the absolute quantification method to qualify the copy gene in each sample using the standard curve as a reference.

Detection limit

Three tenfold dilutions containing 1.8 copies of the *nuc* gene (0.000003 ng/μl), 1.8×10^1 copies of the *nuc* gene (0.00003 ng/μl), and 1.8×10^2 copies of the *nuc* gene (0.0003 ng/μl) were prepared from the reference *S. aureus* genomic DNA extracted from pure culture as well as from artificially inoculated milk. The genomic DNA was quantified by Qubit® 2.0 Fluorometer assay (Invitrogen, USA). Each dilution, both from the two sources was subjected to qPCR assay in ten replicates. The number of positive signals exhibited in each dilution as well as the mean and standard deviation of the Cq were documented. For the dilution that was considered as the limit of detection (positive signals exhibited), the relative standard deviation (RSD) of the DNA measurement of the ten replicates was calculated.

Repeatability (Intra-assay) and reproducibility (Inter-assay)

To assess the precision of qPCR, three dilutions containing 1.8×10^4 copies of the *nuc* gene (0.03 ng/μl), 1.8×10^5 copies of the *nuc* gene (0.3 ng / μl), and 1.8

$\times 10^6$ copies of *nuc* gene (3 ng / μ l) were prepared by Qubit[®] 2.0 Fluorometer assay (Invitrogen, USA). Ten replicates of the three dilutions were subjected to qPCR to determine repeatability whereas three independent assays (by different personnel and time) were performed to document the reproducibility of the qPCR. For all runs, the mean C_q and standard deviations (SD) were calculated.

Primers for the quantitative qPCR assay

Primers (F372/R465) that targeted the *nuc* gene of *S. aureus* recommended by (Alarco'n *et al.*, 2006) for qPCR, were used for the detection and quantification of *S. aureus* from the bulk milk. The oligonucleotide sequence of the primers was verified by the BLAST algorithm in Genbank.

F 5'TGTAGTTTCAAGTCTAAGTAGCTCAGCAA 3', (F372)

R 5'TGCACTATATACTGTTGGTCTTCAGAA 3` (R465). They have expected an amplified product of 94 bp. The amplicon (94 bp) was verified by electrophoresis in 2% agarose gel. They were synthesized in Invitrogen by life technologies (Trondheim, Norway).

Quantitative qPCR amplification condition

All samples (n=92) and the artificially contaminated milk were quantified by qPCR in a duplex. The qPCR was run with the final volume of 20 μ l. The LightCycler[®] 480 SYBR Green I Master mix (Roche, Mannheim, Germany) that contains FastStart Taq DNA polymerase, reaction buffer, d NTP mix, SYBR Green I dye, and MgCl₂ was utilized. Besides, 300 nM of each primer and 2 μ l of genomic DNA were added. The H₂O PCR grade (Roche, Mannheim, Germany) was used to adjust the reaction component to the final reaction volume. In all, qPCR runs the reference *S. aureus* as a positive control, and the PCR-grade H₂O as a negative control was included. All amplification was conducted in LightCycler[®] 480 Instrument (Roche, Mannheim, Germany) in 96 well plates and sealed with sealing foil (Roche, Mannheim, Germany).

The LightCycler[®] 480 Instrument was programmed as follows. Pre-incubation of 1 cycle at 95 °C for 5 min. Amplification of 35 cycles with denaturation at 95 °C for 15 sec., annealing at 60 °C for 1 min. and elongation at 72 °C for 5

sec. *Melting curve* of 1 cycle at 95 °C for 5 sec. 65 °C for 1 min. and continuous at 97 °C and cooling at 40 °C for 10 sec.

Quantitative qPCR results were given as an increase in fluorescence signals of the reporter dye, SYBR Green I, detected and visualized by the LCS480 1.5.1.62 software (Roche, Mannheim, Germany).

At the end of each run, an analysis of melting curves, generated by the LCS480 1.5.1.62 software, was performed to determine the melting point of the amplicons.

The absolute quantification method was applied in this assay. This method depends on the comparison of the C_q value of the samples with the standard curve generated from the amplification of the known/reference *S. aureus* gene. In every qPCR, a new standard curve was included that has relatively similar amplification efficiency. Quantification of the *S. aureus* load in each sample was registered by extrapolating the C_q values of the standard curve which was generated by the light cycler software.

Calculation of the *S. aureus* cell equivalent (SCE) per ml was performed by multiplying the number of copies of the *nuc* gene per qPCR with the ratio of the volume of the DNA containing supernatant (V_s) which is two µl. The initial 4 ml of the milk employed for the extraction of DNA was taken into consideration during the calculation of SCE/ml of milk (Hein *et al.*, 2005).

Data analysis: Microsoft Excel for Windows (MS Office version 8.1 version) was used as a database. Descriptive analysis of the data was conducted with the Microsoft Excel program. The independent sample t-test from The Unscrambler X version X 10.3 software was used to compare the plate count with the qPCR quantification data. Before analysis, all the data were checked for possible outliers. P-value < 0.05 was considered statistically significant.

Results

Standard Curve

In the absolute quantification method of qPCR, the generation of the standard curve is mandatory, as the C_q value of the samples will compare with the standard curve which is generated from the known /reference *S. aureus* gene as previously described.

One of the standard curves generated from a tenfold dilution of 1.8×10^1 to 1.8^5 copies of the *nuc* gene is shown in Fig 1. It has a slope of -3.362, which gives an amplification efficiency of 98.3%. The coefficient of correlation between the Cq value and copy number is calculated as 0.99, which shows the linearity of the quantification.

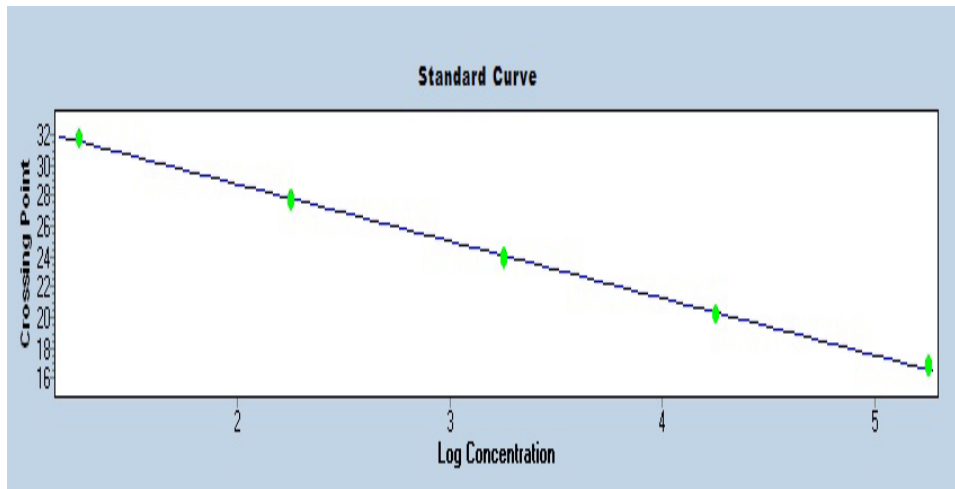


Figure 1. The standard curve generated (Slope-3.36, Y/intercept 38.32, error 0.22) from serial dilution of 1.8×10^1 , 1.8×10^2 , 1.8×10^3 , 1.8×10^4 , 1.8×10^5 copies of *nuc* gene.

Specificity study

The specificity of the primers that targeted the *nuc* gene was studied, with optimized qPCR reaction, on *S. aureus* and other *Staphylococcus* species of dairy isolates. The result showed that the mean Cq value of *S. aureus* was 13.83 ± 0.93 (13.08 ± 0.18 for_ref. *S. aureus*) whereas the mean Cq value for the other *Staphylococcus* species was 30.34 ± 2.65 (Fig.2). The mean melting temperatures of the amplicon for *S. aureus* was 77.49 ± 0.04 °C while for other staphylococcus species was 77.71 ± 0.33 °C.

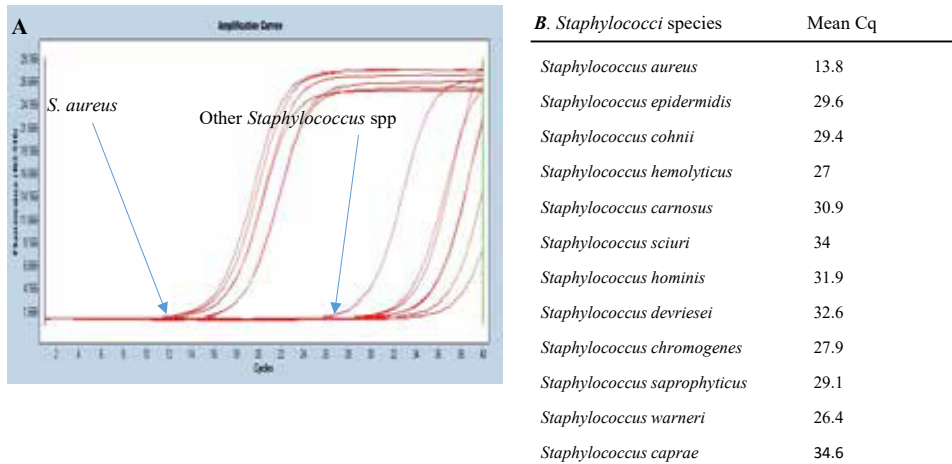


Figure 2. One of the qPCRs runs for specificity study. A. The marked difference in the Cq value of *S. aureus* from the other *Staphylococci* species in the amplification curve of the qPCR assay B. The summarized mean Cq value of *S. aureus* and other *Staphylococcus* spp.

The limit of detection (LOD)

There was no difference in the LOD between the pure culture and milk-extracted DNA. Out of the ten replicates (in both sources) of 1.8×10^2 copies of the *nuc* gene, ten showed positive signal with mean Cq value of 29.09 ± 0.22 . Of the ten replicates containing 1.8×10^1 copies, ten revealed a positive signal with a mean Cq value of 31.98 ± 0.47 . However, in the dilution containing 1.8 copies, only six showed positive signals from pure culture and four from the milk with a mean Cq value of 33.23 ± 0.14 . Therefore, the detection limit of this qPCR assay was 18 copies of the *nuc* gene per PCR reaction with and without the matrix effect. It was also possible to quantify the ten replicates of the 18 copies/qPCR with the assay. The measurement showed a variation of 31.1% as expressed by RSD or the coefficient of variation (CV). Hence, the 18 copies of *nuc* gene /qPCR were not only the detection limit but also a quantification limit of the assay.

Repeatability (Intra-assay) and Reproducibility (inter-assay)

The repeatability (intra-assay) and reproducibility (inter-assay) of the qPCR assay as expressed by the standard deviation (SD) of Cq are presented in Table 1. The SD values range from 0.12 to 0.3 for intra-assay and 0.29-0.59 for inter-assay (all < 1.0 SD) variation, which were in an acceptable range. This showed that the precision of the qPCR assay was good.

Table 1. The mean and SD of three tenfold dilutions of copies of the *nuc* gene subjected to qPCR to determine the intra-assay and inter-assay variation of the assay

	Number of replicates N	1.8 X 10 ⁴ copies		1.8 X 10 ⁵ copies		1.8 X 10 ⁶ copies	
		Mean Cq	SD	Mean Cq	SD	Mean Cq	SD
Intra-assay	10	21.8	0.30	18	0.17	14.8	0.12
Inter-assay	3	22.3	0.59	18.22	0.32	14.8	0.29

CFU and R² of artificially contaminated milk

In log-transformed data, the mean SCE count (5.9 log SCE/ml) obtained by qPCR was higher than the mean CFU count (5.3 log CFU/ml) obtained by the plate count. The coefficient of correlation (R²) between the log counts of SCE / ml and CFU/ml was 0.9578, which showed that it is possible to calculate the number of colonies forming the unit of *S. aureus* in raw milk by running the qPCR.

Comparison of plate count data (CFU/ml) and qPCR (SCE/ml) data of naturally contaminated raw milk

There was no sample below the quantification limit of the assay. The mean quantification value was 5.85 +/- 1.55 log SCE/ ml and 4.46 +/- 1.06 log CFU/ml in the qPCR and the plate count, respectively. The median of the counts was 5.59 log SCE/ml and 4.8 log CFU/ml for qPCR and plate count, respectively. There was a statistically significant difference (p < 0.05) in counts of *S. aureus* between the two methods.

Classification of the two methods by *S. aureus* count level

Classification of the two methods based on the two categories of counts thus: ≤ 5 log /ml *S. aureus* counts and > 5 log/ml of *S. aureus* count has been ana-

lyzed. The results showed that, out of the total 92 samples, 59 (64%) samples, *S. aureus* > 5 log SCE/ml were counted by qPCR assay whereas > 5 log CFU/ml of *S. aureus* were counted only from 33 (36%) of the samples by plate count method. Twenty-six (28 %) samples, which were considered as having < 5 log/ml of *S. aureus* count with the plate count method, were found to have > 5 log/ml count with qPCR assay.

Discussion

The present study evaluated the performance of qPCR, targeting the *nuc* gene and using Syber Green 1 as a dye, for quantification of *S. aureus* in raw milk and compare the results with the conventional plate count method. The overall result showed that the qPCR assay is more sensitive and specific than the plate count method in the qualification of *S. aureus* in raw milk.

In the present study, the primers showed good specificity that can differentiate *S. aureus* from other *Staphylococcus* species due to low C_q value. This finding is in agreement with Alarco'n *et al.* (2006) and Brakstad *et al.* (1992).

The sensitivity of the qPCR both from pure culture and from milk was 18 copies /PCR reaction. Although the food matrixes have a direct effect on the sensitivity of an assay (Hein *et al.*, 2001a), however, it was not evident in the present study. The reason may be associated with efficient DNA extraction procedures. A good DNA extraction procedure could significantly improve the sensitivity of PCR assay as reported by Kim *et al.* (2001) in the detection of *S. aureus* from mastitis milk. Alarco'n *et al.* (2006) demonstrated a detection limit of 10-20 cells of *S. aureus* from food by qPCR using aSYBR-Green I as detection chemistry, which is comparable with our study.

The repeatability and reproducibility of the qPCR assay were in the acceptable range, which showed the good precision of the qPCR assay. Variation in the qPCR results could be occurred due to temperature variation that could affect the annealing and/or denaturation of the DNA, or due to template concentration variation introduced by pipetting error and stochastic variation (Bustin *et al.*, 2009).

There was a statistically significant difference ($p < 0.05$) in the qualification of *S. aureus* in the milk between the two assays. The qPCR assay showed > one

log higher count than the plate count method in the naturally contaminated method. In consistence with this observation, other research also reported higher *S. aureus* count in naturally contaminated raw milk (Hein *et al.*, 2005; Studer *et al.*, 2008) by qPCR assay than the plate count method. Postollec *et al.* (2011) reviewed the following possible reasons for the higher bacterial count in the culture-independent molecular method than in the culture-dependent method. The presence of intact DNA from dead cells as well as the presence of viable but non-cultivable bacteria, which can only be detected by molecular method, could increase the counts. Moreover, one CFU on the plate may be originated from more than one cell, and, in addition, some primers involved in the molecular assay may target several multi-copy genes (e.g. 16S rRNA) that could increase the count.

The detection of dead *S. aureus* in food is a public health concern. It helps for retrospective analysis of the level of contamination starting from the production up to that point in time, which is important for the microbial risk assessment study (Hein *et al.*, 2005). In the present study 28 % of the samples, which were considered as having < 5 log/ml *S. aureus* count with the plate count method, were found to have > 5 log /ml count with qPCR, which indicated the high sensitivity of the molecular method. Samples with > 5 log /ml count of *S. aureus* may perform SE if the *S. aureus* population has enterotoxigenic potential and yet the SE could stay biologically active for a long period as SE is thermostable and resistant to enzymatic activities, although the bacteria it produced is dead (Hennekinne *et al.*, 2011). A recent study in the same area reported that 51 % of dairy isolates of *S. aureus* have enterotoxigenic potential (Tarekgne *et al.*, 2016). Taking these facts, it is relevant to suggest that there is a high risk of an SFP outbreak in the study area.

In comparison with the plate count method, the level of contamination in qPCR is minimum even better than the conventional PCR (use of gel electrophoresis) because the data are generated in real-time immediately after amplification. People working in the qPCR do not afraid of contamination by *S. aureus* and other pathogens as they are mostly working with dead DNA. Furthermore, in Ethiopia where clinical and subclinical mastitis is a major dairy cow problem (Duguma *et al.*, 2014; Tolosa *et al.*, 2015) monitoring, of the etiological agents with the qPCR, is preferable as the conventional plate count methods are costly and time-consuming. The qPCR has been reported as an important and efficient alternative to the plate count method in monitoring inframammary infections elsewhere (Graber *et al.*, 2007; Studer *et al.*, 2008). Although it did

not perform in the current study, it is also possible to multiplex the assay for simultaneous detection and quantification of more than one pathogen in food.

One limitation of the primers, observed in the present study is that when the level of *S. aureus* in the samples is low, the Cq value becomes high which could overlap with the Cq value obtained from high levels of other staphylococcus species (data not shown). To minimize this problem, during the optimization of the current qPCR assay, we reduced the qPCR amplification cycle from 40 to 35 cycles.

The current study showed us the following advantages and prospects of qPCR over the conventional plate count method. Due to its automated nature, the qPCR generates quantified results within a short period, which is practically important during the diagnosis of food poisoning outbreaks such as SFP. Not only the speed but gives also quantified data of viable and dead *S. aureus* cells in the samples, which shows the high sensitivity of the assay. From a microbial risk assessment perspective such quantified and sensitive data are so important to characterize and quantify the microbial risks that influence food safety (Postollec *et al.*, 2011).

Conclusions

The present study showed good comparative performance between qPCR and culture-based plate count methods for the quantification of *S. aureus* in raw milk. In light of these findings, the author strongly recommended the use and application of qPCR assay for routine and research work for its advantage of rapid, sensitivity, and reliability. Further study, on validation of the qPCR protocol in different food types for quantification of the common foodborne pathogens and assessing the cost-benefit analysis of the assay are required.

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