



Development of a PCR-TTGE assay for rapid detection of *Staphylococcus* species in processed meat products

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Abstract

Some *Staphylococcus* species are believed to be the main cause of bacterial infections and foodborne outbreaks. Several reports have discussed the enterotoxigenic properties of some *Staphylococcus* species, but due to the shortage of efficient diagnostic techniques, most studies have focused only on *Staphylococcus aureus*. Thus, developing a culture-independent, selective, and rapid detection method for *Staphylococcus* species in food products is of great importance. In this study, PCR-amplified *tuf* gene sequences were assessed by temporal temperature gradient gel electrophoresis (TTGE) in order to detect and differentiate between different *Staphylococcus* species in Iranian food samples. The PCR sensitivity and specificity were evaluated against DNA samples extracted from six *Staphylococcus* species, including *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. intermedius*, *S. chromogenes*, and *S. hominis*, using a commercially available kit and a cost-effective, rapid, non-commercial boiling method. Using the boiling method, the sensitivity of the *tuf* PCR was 9×10^1 CFU/mL for the salami samples spiked with *S. aureus*, ten times less sensitive than the commercial kit. After optimizing the TTGE conditions, a species-specific TTGE pattern was obtained based on the differences between the amplified sequences from various species. This TTGE pattern was applied to detect *Staphylococcus* species in food samples from the market. The presence of *Staphylococcus* species was confirmed in 6 out of 10 tested salami products. The results demonstrate that the PCR-TTGE method is an alternative method that may be specific and sensitive enough to assess the presence of possible *Staphylococcus* contamination in meat processed food samples. More studies using different food samples should be considered for an in-depth analysis of bacterial contamination.

1. Introduction

Staphylococcus bacteria are naturally present in the skin and mucosal membranes and can also be isolated from a wide range of

contaminated foods (meat, cheese, or milk) and natural resources (soil, sand, dust, air, or water). Due to pathogenic activities, reliable methods for identifying *Staphylococcus* at the genus and species level are of significant

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importance. In this regard, various methods including culture-dependent and culture-independent methods have been developed to detect *Staphylococcus* species in foods (Gill, 2017; J. Hennekinne & Le Loir, 2014; Wang & Salazar, 2016).

Currently, culture-dependent methods are applied for bacterial identification. However, associated obstacles such as 1) being time-consuming (3–5 days) and laborious, 2) difficulty in bacterial isolation from natural sources and culture in specific media, 3) lack of knowledge about the real conditions that most bacteria grow, and 4) difficulty in differentiation between coagulase-negative staphylococci require emphasis on the development of novel techniques to detect the bacterial species (Foddai & Grant, 2020; Fusco & Quero, 2014; Kloos & Schleifer, 1975).

Culture-independent methods, such as nucleic acid-based approaches that can amplify the target DNA/RNA to a detectable level within a few hours, are powerful tools for the detection of foodborne pathogens (Wang & Salazar, 2016). Some specific primers for the detection of *Staphylococcus* species, including *S. aureus* (Bogestam, Vondracek, Karlsson, Fang, & Giske, 2018), *S. epidermidis* (Kord et al., 2018) and *S. saprophyticus* (de Paiva-Santos, de Sousa, & Giambiagi-deMarval, 2018), have been reported. However, their application is limited to only one specific species in each test. Several studies have reported a multiplex PCR assay for diagnosing different species in one reaction using multiple oligonucleotides, but this technique has the limitation of designing proper primers based on the prior knowledge of target sequences. It is also difficult to optimize PCR conditions for the number of primers needed to identify several species in one reaction (Kim, Hong, Lim, Heu, & Roh, 2018). Some other PCR-based methods, like real-time PCR, are standard procedures in

many laboratories. But there also have drawbacks such as prior knowledge of gene sequences and designing several specific primers for some *Staphylococcus* species. So, these methods are insufficient for detecting food contaminations, which often involve a mixed population of *Staphylococcus* species.

Consequently, gradient gel electrophoresis (GGE) has been introduced, in which the gene of interest can be amplified without pre-identification of the target in a mixed population. The advantages this method has compared to other traditional approaches are: it does not require 1) time-consuming processes like conventional and biochemical techniques, 2) differential media for isolation of *Staphylococcus* species, and 3) substantial labor-intensive techniques such as hybridization probes and gene sequencing (Al-Mailem, Kansour, & Radwan, 2017).

In this study, we developed a temporal temperature gradient gel electrophoresis (TTGE)-based PCR to monitor the presence of various *Staphylococcus* species in Iranian meat products for the first time. For this purpose, the specific primers targeting *tuf* gene, encoding elongation factor Tu, were used. This gene shows a high degree of polymorphism among *Staphylococcus* species making it a suitable candidate for detecting *Staphylococcus* contaminations using the TTGE method (Hwang, Kim, Park, Song, & Kim, 2011; Iversen et al., 2020; Khosravi et al., 2018).

2. Materials and Methods

2.1. Reagents and Chemicals

All reagents and chemicals used in the preparation of electrophoretic gels and silver staining were obtained from Merck Co. (Germany). 100 bp DNA Ladder and loading dye were obtained from Fermentas Co. (Canada). The forward and reverse primers were purchased from Macrogen Co. (South Korea). Ethidium bromide and all reagents

used in PCR were obtained from CinnaGen Co. (Iran).

2.2. Bacterial Species

The staphylococcal species used in this study, including *S. aureus* (PTTC: 1431), *S. epidermidis* (PTTC: 1435), and *S. saprophyticus* (PTTC: 1440), were purchased from the Persian Type Culture Collection (PTCC, Iran). In addition, two clinical isolates representing two different species of *Staphylococcus* (*S. intermedius* and *S. chromogenes*) and one food isolate obtained from cheese (with 99% similarity with *S. hominis* based on 16S rDNA sequencing) were used.

All species were cultured on non-selective Tryptic soy broth (TSB) under aerobic conditions at 37°C for 24 h.

2.3. Rapid DNA Extraction

DNA was extracted from isolated bacteria using the boiling method with some modifications (Pui et al., 2011). Aliquots of 1 mL of 24 h cultured bacteria were centrifuged at 15000 g for 10 min, and the pellets were resuspended in 500 µL sterile deionized distilled water. The suspensions were boiled for 10 min to release DNA from bacteria.

In addition, the efficiency of the two different DNA extraction methods, the BIONEER DNA extraction kit (AccuPrep®, BIONEER Corporation, Korea) and the boiling method, was compared through the extraction of DNA from food samples spiked with *S. aureus*.

To optimize DNA extraction from salami, a pre-enrichment step was performed by incubating 1 g of the homogenized salami samples in 9 mL Buffered Peptone Water (BPW) at 37°C for 24 h. The DNA was then extracted and stored at -20°C.

2.4. PCR Amplification of the *tuf* gene

For *Staphylococcus* identification, the *tuf* gene (GeneID: 45573819) was amplified using TStaG422f (5'- GC clamp GGC CGT GTT GAA CGT GGT CAA ATC A-3') and TStag765r (5'-TIA CCA TTT CAG TAC CTT CTG GTA A-3') specific primers (Martineau et al., 2001). The reverse primer "I" indicates nucleotide analog inosine. For TTGE experiments, the forward primer contains a 40 bp GC-clamp (CGCCCGCCGCGCCCGCGG CCGTCCCGCCGCCCCCGCCCG).

DNA extracts from the pure bacterial cultures and spiked foods were subjected to PCR and then analyzed for the TTGE pattern. The PCR reaction mixture (20 µL) contained 1 U/µL *Taq* DNA polymerase, 1 µL of each primer (10 pmol), 0.1 mM dNTPs, 1 mM MgCl₂, and 2 µL buffer (10X). Positive (*S. aureus*) and negative controls were included in each reaction. The amplification condition was as follows: 94°C for 5 min, followed by 39 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 20 s, and 72°C for 7 min for final elongation. The PCR products were detected using 1.5% agarose gel electrophoresis.

2.5. Specificity of the *tuf* PCR

The specificity of the PCR assay was verified using purified genomic DNA from *Staphylococcus* species and some important foodborne bacteria, including *Escherichia coli* ATCC: 25922, *Klebsiella pneumoniae* ATCC: 700603, *Salmonella typhimurium* ATCC: 14028, and a clinical isolate (*Proteus mirabilis*).

2.6. Sensitivity of the *tuf* PCR

The overnight *S. aureus* culture was serially diluted to 10⁷ CFU/mL to determine the PCR sensitivity. The *S. aureus* initial concentration was estimated by the viable count method as

well as absorbance comparison with the McFarland standard table.

To determine the limit of detection (LOD) in artificially-contaminated food samples, 100 μL of each dilution prepared from *S. aureus* bacteria was added to a homogeneous salami mixture containing 1 g salami and 9 mL BPW medium (Kumar, Murali, & Batra, 2009). Next, 1 mL of each dilution was transferred to a 1.5 mL microtube, and DNA was extracted using the boiling method and a BIONEER kit after 24 h of incubation. The PCR reaction was then performed for each dilution. Negative control and unspiked food samples were included in every step.

2.7. Temporal Temperature Gradient gel Electrophoresis Analysis (TTGE)

After PCR, TTGE assay was applied to analyze the amplicons. TTGE was performed using the DCode universal mutation detection system (Bio-Rad Laboratories, USA). The gels were 16 \times 16 cm by 1 mm (60 mL) composed of 6% (w/v) acrylamide: bisacrylamide (37.5:1), 6 M urea, 80 μL of N,N,N',N'-tetramethylethylenediamine (TEMED), and 0.1% (w/v) ammonium persulfate. Gels were electrophoresed in 1 \times Tris–acetate–EDTA (TAE) buffer at pH 8.4.

The TTGE running conditions in terms of migration time, voltage, and temperature range were optimized with six species of *Staphylococcus* as the reference bacteria. The best separation of the standard *Staphylococcus* species in spiked salami samples was obtained at 130 V and a temperature gradient from 55°C to 61°C for 5 h (1.2°C/h). A diversity ladder was made with a mixture of six *Staphylococcus* PCR products. The migration pattern of the TTGE bands was observed with silver staining (Chevallet, Luche, & Rabilloud, 2006).

2.8. TTGE-based Detection of *Staphylococcus* Species in Salami Samples

Ten samples, including nine packed and branded salami and one hand-made salami, were randomly selected from local markets in Mashhad. A similar procedure described in Section 2.6 was used for DNA extraction from the food samples. Detection of *Staphylococcus* species in 10 purchased salami samples was performed under the TTGE condition described in Section 2.7.

3. Results and Discussion

The recognition of pathogens in food samples is a crucial step in the management of food safety. Although culture-dependent methods are regarded as the gold standard in microbiological assays, they are time-consuming and can easily be cross-contaminated. Thus, developing a molecular approach that provides a simple, rapid, and accurate assay could be a step forward. TStag422f/TStag765r primers targeting *tuf* have been used to investigate the differences in the sequences of PCR products and to check the possibility of using this gene for distinguishing the *Staphylococcus* species using TTGE in milk samples (Filleron et al., 2014). In recent years, variable sequences of the *tuf* gene have been used to distinguish *Staphylococcus* species using the PCR-DGGE. This technique is sensitive, but the use of chemicals to create an electrophoretic slope in DGGE limits its application (H.-C. Chen & Hwang, 2008; Kassem, Esseili, & Sigler, 2011). As mentioned above, the application of *tuf*-PCR-TTGE to detect bacteria in milk products was previously done (Filleron et al., 2014), but applying it to solid foods with high amounts of inhibitory materials (especially meat products) remains a challenging process. Considering these complications, this study optimized the *tuf*-PCR-TTGE to differentiate *Staphylococcus* species in a meat product.

3.1. Specificity and Sensitivity of *tuf* PCR

Genomic DNAs of the six standard *Staphylococcus* species were extracted using the boiling method and used in a PCR reaction with 39 cycles. A 380 bp fragment of the *tuf* gene was observed on 1.5% agarose gel (Fig 1. A). To examine the specificity of TStag422f/TStag765r primers among several species of *Staphylococcus*, some non-*Staphylococcus* foodborne bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Proteus mirabilis* were tested by the same PCR method. The PCR analysis of these bacteria showed no detectable bands (Fig 1. B).

The use of a specific pair of primers, TStag422f/TStag765r in our study, showed high specificity in the amplification of the *tuf*

gene from *Staphylococcus* species. However, in Martineau et al.'s report in 2001, TStag422f/TStag765r primers also amplify the *tuf* gene in *Macrococcus caseolyticus* and *Enterococcus faecalis*. As nonspecific efficacy is 1000 or 10000 less than specific amplification efficacy, it is unlikely to influence our results during the identification of the *Staphylococcus* species. In 2004, Morot-Bizot et al. showed that TStag422f/TStag765r primers do not amplify the *tuf* gene in *M. caseolyticus*.

To test the LOD and the *tuf* PCR sensitivity, serial dilutions of *S. aureus* were made, and DNA was extracted from each dilution with the boiling method. As shown in (Fig 1-C), the sensitivity of the *tuf* PCR with 39 cycles for *S. aureus* was 9×10^4 CFU/mL.

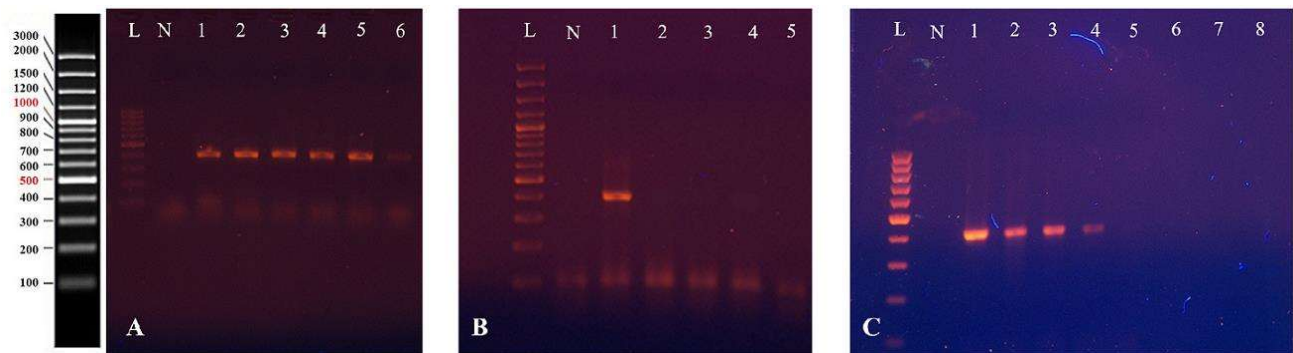


Figure 1: PCR products of the *tuf* gene amplified with TStag422f and TStag765r primers in:

A) *Staphylococcus* standard species. Representative lanes from left to right: L)100 bp DNA ladder (Fermentas, USA), N) Negative control, 1) *S. aureus* PTCC 1431, 2) *S. saprophyticus* PTCC 1440, 3) *S. epidermidis* PTCC, 4) *S. chromogenes* (clinical isolate), 5) *S. intermedius* (clinical isolate), and 6) *S. hominis* (food isolate).

B) Non-*Staphylococcus* standard species. Representative lanes from left to right: L)100 bp DNA ladder (Fermentas, USA), N) Negative control, 1) Positive control, 2) *Escherichia coli* ATCC 2592, 3) *Klebsiella pneumoniae* ATCC 700603, 4) *Salmonella typhimurium* ATCC 1402 and 5) *Proteus mirabilis* (clinical isolate)

C) Various *S. aureus* dilutions (9×10^7 - 9×10^0 CFU/mL) to observe the sensitivity of PCR assay for detection of *Staphylococcus* spp. Representative lanes from left to right: L) 100 bp DNA ladder (Fermentas, USA), N) Negative control, and 1, 2, 3, 4, 5, 6, 7 and 8 dilutions of 9×10^7 CFU/mL to 9×10^0 CFU/mL, respectively.

The comparative results of the cost-effective boiling method and the BIONEER commercial kit to extract DNA from artificially-contaminated food samples demonstrated that the BIONEER kit was 10x more sensitive than the boiling method (Fig 2. A and B). However, considering the fact that the boiling method was 100x cheaper than the kit and both sensitivities were below the unsatisfactory range (Council, 2003; Gilbert et al., 2000), setting up a cheaper approach would help to gain industry-customer confidence and be more easily commercialized for screening large amounts of meat foodstuffs. The sensitivity of the PCR assay was 9×10^1 CFU/mL when DNA was extracted from salami samples spiked with different dilutions of *S. aureus* enriched with BPW using the boiling method (Fig 2. B).

The sensitivity of PCR for *Staphylococcus* DNA extracted by the boiling method in different studies is inconsistent in various reports. While some report good sensitivity for their cases (Berrada, Soriano, Mañes, & Pico, 2006; Chiang et al., 2012; Hagen et al., 2005), there are others who have observed similar (L. Chen et al., 2009) or even less sensitivity in comparison to our experiments (Wilson, Cooper, & Gilmour, 1991). Still, others have also reported a lower sensitivity of the PCR test in spiked bacteria samples compared to our method (Gandra, Fernandez, Silva, & Silva, 2016; Ramesh, Padmapriya, Chrashekar, & Varadaraj, 2002; Wei et al., 2019). However, several studies have reported similar results (Arunrut, Kiatpathomchai, & Ananchaipattana, 2018; Botaro et al., 2013; Kumar et al., 2009) or higher sensitivity compared to our method (Fricker, Messelhäuser, Busch, Scherer, & Ehling-Schulz, 2007; Nakano et al., 2004; Trnčíková, Hrušková, Oravcová, Pangallo, & Kaclíková, 2009)

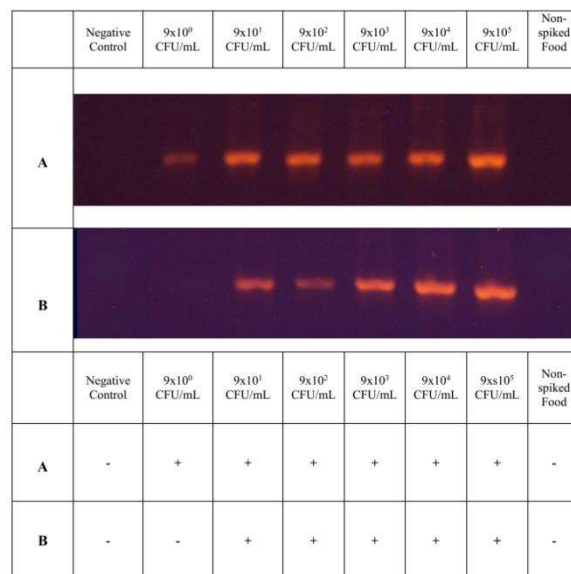


Figure 2. PCR assay sensitivity for detection of *Staphylococcus* spp. in salami samples spiked with different concentrations of *S. aureus* (9×10^5 CFU/mL to 9×10^0 CFU/mL). The results were tested with DNA extracted from 24-h pre-enrichment spiked salami samples using (A) a BIONEER kit and (B) the boiling method.

3.2. Temporal Temperature Gradient Gel Electrophoresis Analysis

Denaturing electrophoresis, such as PCR-TTGE/DGGE, has been introduced as a suitable technique to provide a comparative bacterial DNA fingerprint to study food safety (Besharati, Bahrami, Mashreghi, Matin, & Bahrami, 2017; Ercolini, 2004; Garofalo et al., 2017). However, these fingerprint methods also have some drawbacks. One limitation is the separation of relatively short DNA fragments (300-400 bp) which affects the design of primers and probes for analysis. Moreover, in closely related organisms such as different species of *Staphylococcus*, separation of fragments with different sequences is not always possible because of a similar melting profile of the fragments (Adil, 2015). Several studies have used PCR-DGGE/TGGE for 16S rDNA to detect microbiota diversity, although the accuracy of this approach for identifying the species has been questioned (Dahllöf, Baillie, & Kjelleberg, 2000). The similarity in the *tuf* gene sequence has been reported among 29 species of *Staphylococcus* in a range of 86% to 97%, which

is less than the similarity in the 16S rDNA gene (90% to 99%). For this reason, *tuf* was suggested as a potential species-specific marker for the *Staphylococcus* genus (Bergeron et al., 2011; H.-C. Chen & Hwang, 2008; Hwang et al., 2011; Khosravi et al., 2018). Therefore, the *tuf* encoding the elongation factor Tu with the highest polymorphism(s) among different staphylococci was selected in this study.

The TTGE was conducted at 130 V with a temperature gradient from 55°C to 61°C for 5 h (1.2°C/h). The PCR amplification products from different *Staphylococcus* species migrated to distinct distances and successfully differentiated (Fig 3). This indicates a sufficient variation in the *tuf* gene sequence to discriminate between different *Staphylococcus* species. Letters a-f represent the species' respective bands. To construct the *Staphylococcus* diversity ladder, amplicons of standard species were mixed, and the pattern was applied as a diversity ladder for TTGE analysis of *Staphylococcus* bacteria in food samples.

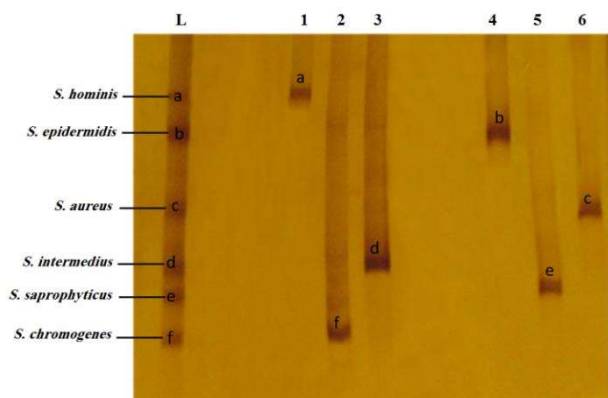


Figure 3. Temporal temperature gradient gel electrophoresis band separation patterns of *tuf* PCR products in *Staphylococcus* standard species. Each lane represents: L) Diversity marker, 1) *S. hominis* (food isolate), 2) *S. chromogenes* (clinical isolate), 3) *S. intermedius* (clinical isolate), 4) *S. epidermidis* PTCC 1435, 5) *S. saprophyticus* PTCC 1440, and 6) *S. aureus* PTCC 1431.

Moreover, the results showed that the three *Staphylococcus* species in the artificially contaminated salami samples migrated to the equivalent distances of the same species (Figure

4). TTGE patterns during different runs showed good reproducibility.

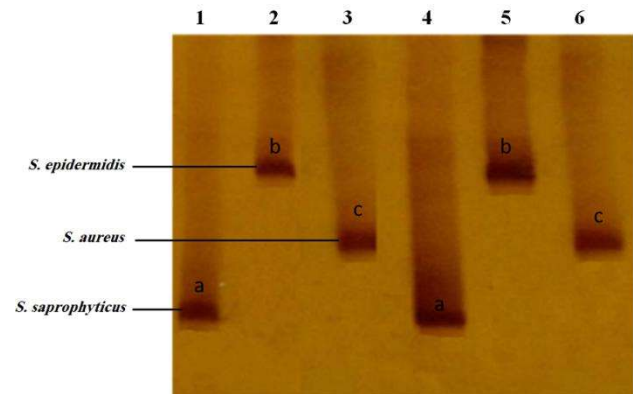


Figure 4. Temporal temperature gradient gel electrophoresis band separation patterns of *tuf* PCR products in food samples spiked with *Staphylococcus* species. Each lane represents: 1) *S. saprophyticus* PTCC 1440, 2) *S. epidermidis* PTCC 1435, 3) *S. aureus* PTCC 1431, 4) food sample inoculated with *S. saprophyticus* PTCC 1440, 5) food sample inoculated with *S. epidermidis* PTCC 1435 and 6) food sample inoculated with *S. aureus* PTCC 1431.

3.3. TTGE Analysis of *Staphylococcus* species Contamination in Salami Samples

A total of 9 packed and branded salami samples and one hand-made salami sample were randomly selected, and the presence of *Staphylococcus* species was investigated. For this purpose, DNA extraction was carried out using the cost-effective, easy, and efficient boiling process with a 24-h enrichment step in the BPW medium, and then *tuf* gene amplification was performed by PCR method. Observation of a band with a size of about 400 bp indicates the presence of the *Staphylococcus* genus in the samples. As shown in (Fig 5), the presence of the *Staphylococcus* genus was confirmed in 6 out of 10 tested salami products using *tuf*-PCR.

Among all samples, the presence of the *Staphylococcus* genus was indicated in sample no. 9 before and after enrichment (time: 0 and 24 h). This difference could be due to the high range of diverse bacteria population levels as it was an unidentified and hand-made sample without any standardized protocols. A 24-h pre-

enrichment step facilitates bacterial growth to a detectable range. Also, food inhibitors are reduced by dilution in the pre-enrichment medium. So, the pre-enrichment can

significantly improve the sensitivity of the method to identify even a low population of bacteria in packed and hand-made products.

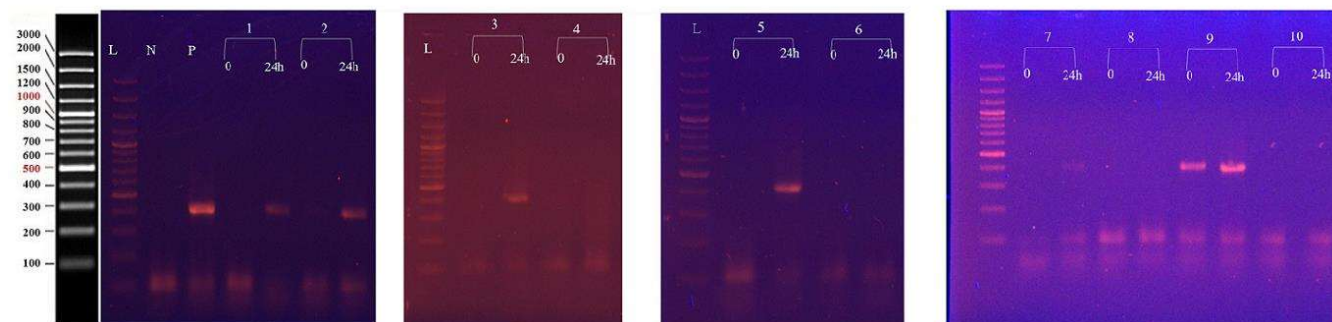


Figure 5. PCR Products of the *tuf* gene amplified with TStaG422f/TStag765r primers in prepared salami samples from the market. Each lane represents: L) 100 bp DNA Ladder (Fermentas, USA), N) Negative control, P) Positive control, Lane 1–10) Salami no.1 to salami no. 10 at zero and after 24 h of pre-enrichment.

* Salami no. 9 is a hand-made salami; the others belong to branded and prepacked products.

All these samples were differentiated into separated bands by PCR-TTGE. As shown in (Fig 6), by matching the TTGE pattern of every unspiked salami sample with the diversity marker, two samples (no. 2 and no. 5) showed parallel bands with the *S. intermedius* and *S. hominis* in the diversity ladder. It was revealed that *S. hominis* species was present in salami sample no. 2 (represented in lane 2). Another band was in salami no. 5 (represented in lane 4), consistent with the standard *S. intermedius* in the diversity ladder.

no. 2, 2) 24-h pre-enriched salami no. 2, 3) 24-h pre-enriched salami no. 3, 4) 24-h pre-enriched salami no. 5, 5) 24-h pre-enriched salami no. 7, and 6 and 7) Hand-made salami no. 9 at zero and after 24 h of pre-enrichment, respectively.

The authenticity of the PCR product of the *tuf* gene from the test sample (Salami no. 5) was assessed by direct sequencing. The local similarity search using the Basic Local Alignment Search Tool revealed 99% similarity with *S. pseudintermedius*, *S. intermedius*, and *S. delphini*. These three bacteria are very similar, except for the *Staphylococcus intermedius* Group (SIG) (Ben Zakour, Beatson, van den Broek, Thoday, & Fitzgerald, 2012). These results are consistent with the TTGE observations.

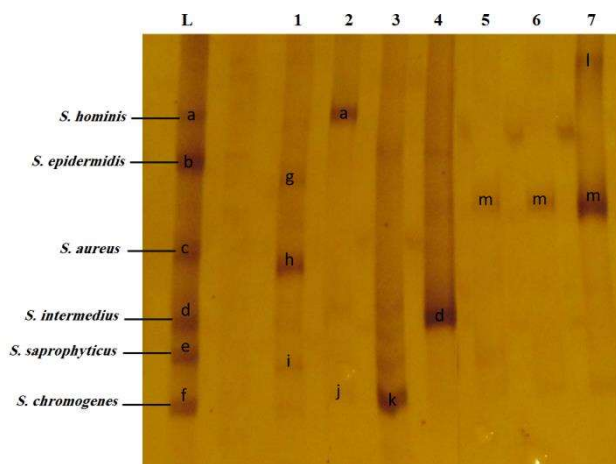


Figure 6. Matching the TTGE band pattern of PCR products from foods prepared from the market. The columns from left to right represent: L) Diversity marker, 1) 24-h pre-enriched salami

As discussed earlier, this is the first report of *tuf*-PCR-TTGE for the identification of *Staphylococcus* species in a complex structure meat product. The proposed molecular approach efficiently differentiated *Staphylococcus* species from each other in our study and was also successful in detecting the salami samples contaminated with *S. intermedius* and *S. hominis*. Identification of the *S. intermedius* is

very important as different strains of this species are involved in the production of disease-causing enterotoxins (Futagawa-Saito et al., 2004; J.-A. Hennekinne et al., 2010).

4. Conclusion

In conclusion, using PCR-TTGE as a selective diagnostic technique for *Staphylococcus* species in contaminated meat products suggest that this method can be applicable to food safety. Furthermore, some optimization in our method, such as 24-h pre-enrichment of samples, can offer an alternative, sensitive, and cost-effective way to screen the presence of possible Staphylococcal contaminations in foods at low levels. To the best of our knowledge, this optimized *tuf*-PCR-TTGE has not been previously applied to assess *Staphylococcus* diversity in meat products. For greater accuracy, we need to achieve a species-specific TTGE profile with more standard species and also analyze a broader range of food products.

Abbreviation

HPV: Human papillomaviruses; MHC: Major histocompatibility complex; HLA: Leukocyte antigen; PC: Population coverage

Conflict of Interest

The authors declare that they have no competing interests.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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