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Screening of Ready-to-Eat Canned Fish from Malaysian Supermarkets to Observe the Occurrence of *Staphylococcus aureus* using MPN-Duplex PCR

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Abstract

Staphylococcus aureus is recognized as a major human pathogen causing foodborne diseases worldwide. Since no further cooking is required prior to consumption of Ready to Eat (RTE) canned fish, their safety and microbiological quality are often questionable. The present study aims to detect and quantify *S. aureus* by MPN-PCR technique in order to have an idea about possible contamination of *S. aureus* in RTE canned fish sold in Malaysian markets. In this study, a combined method of the Most Probable Number (MPN) and duplex Polymerase Chain Reaction (PCR) of *S. aureus* with Internal Amplification Control (IAC) was developed to detect and enumerate *S. aureus* species in the canned fish samples. A total of 62 canned fish samples were analyzed, and presence of the target bacteria was confirmed in 52% (32) samples yielding 270 bp PCR product from *nuc* gene. The microbial loads of *S. aureus* in the tested samples were 9.50 x10^{-1} MPN/g to <4.41 x10^{3} MPN/g (borderline levels) resulting from poor handling, processing and temperature control. The optimized duplex MPN-PCR method can facilitate routine screening and quantification of *S. aureus* at any stage in food supply chain. Determining the occurrence and quantification of *S. aureus* in RTE canned fish may help promote the awareness toward RTE canned food and improve current practices of manufacturing, canning and post-canning processes in Malaysia.

Keywords: Canned Fish*; Staphylococcus aureus*; RTE Food; Microbial contaminants; MPN-duplex PCR

Introduction

In order to maintain vital functions of our body, production and consumption of safe and healthy food are necessary. With the emergence of new technology and increase of food varieties, food related problems are increasing day by day worldwide especially due to incapability to ensure food safety both in production and consumption levels. Production of food products maintaining proper hygiene as well as presenting those for consumption without interrupting the standard hygiene procedure is crucial [1].

Each year almost 1 in 10 people become ill due to consumption of contaminated food, as estimated by World Health Organization [2]. Among other bacterial pathogens, *S. aureus* has been recognized as a major human pathogen causing foodborne diseases worldwide [3,4]. Moreover, *S. aureus* is used as an indicator organism in the food and pharmaceutical industries to evaluate compliance to Good Manufacturing Process (GMP) [5].

The term Ready-to-Eat (RTE) foods refer to prepared food products which do not need further thermal processing before consumption. RTE food products which are instantly available are a strong source of essential nutrients for consumers and canned fish, for instance, canned tuna is the second most consumed seafood in the U.S., acclaiming hundred percent pathogen free RTE canned seafood [6]. However, the safety and microbiological quality of RTE foods are often questionable, since no further cooking or processing is accomplished before consumption and the presence of any pathogenic bacteria in food might pose a microbial hazard to consumers' health [7,8]. An optimal thermal process results in destruction of pathogenic (disease-causing) bacteria and control spoilage organisms including *S. aureus* in food products [9]. Therefore, the presence of *S. aureus* in RTE canned fish indicates ineffectually maintained GMP in production area or canning process.

The culture-based methods that have been conventionally used for bacteria detection are time-consuming, labor-intensive, and non-specific [10]. A number of PCR-based techniques have been widely used for the fast detection of bacterial species [11,12]. However, these techniques failed to successfully estimate the concentration of *S. aureus* as well as to differentiate the presence of live and dead cells while, the MPN method can efficiently enumerate the viable microbial load of target organisms. Moreover, the MPN method could be made more sensitive and effective through the use of a large volume (up to 25 ml) of inoculum in a number of enrichment steps [13] thereby allowing the detection of small numbers of bacteria using PCR assay. Luan et al. (2008) [14] reported that MPN-PCR method offers about 100 times more sensitivity than the direct PCR technique.

In recent years, several studies have reported the contamination *of S. aureus* in sushi and/or sashimi [15-18]. However, there is insufficient information regarding the presence of *S. aureus* strains in RTE canned fish and to the best of our knowledge, this is the first report of MPN-PCR to detect and quantify *S. aureus* as an indicator of possible contamination in RTE canned fish. The present study aims to gain an insight about the occurrence and quantity of *S. aureus* in RTE canned fish sold in Malaysian markets.

Materials and Methods

Sample processing

A total of 62 RTE canned fish were purchased from different supermarkets in Kuala Lumpur and Selangor, Malaysia and transported to laboratory for processing within an hour.

Sample enrichment

The samples were enriched by following the protocol adopted by Puah et al. [19], with modifications. Twenty-five grams of each fish sample was mixed with 225 ml of Buffered Peptone Water (BPW; BactoTM, France) followed by homogenization and pre-enrichment by incubating at 37 ˚C for 6 h. After pre-enrichment, all the samples were diluted from 10^{-1} to 10^{-7} in Tryptic Spy Broth (TSB; Oxoid, UK) containing 7.5% NaCl. One ml aliquot was taken from each of the diluted samples and transferred into 1.5 ml sterile microfuge tubes in triplicates. These tubes were incubated at 37 ˚C for 14 to 16 h and then stored until further analysis.

DNA extraction

The boiled cell lysis method [20] was used to extract the crude DNA from the turbid tubes. In brief, 1 ml of food homogenate was centrifuged in a 1.5 ml microfuge tube at 12,000 rpm for 3 min. After discarding the supernatant, the pellet was re-suspended in 100 µl of 1X PBS buffer at pH 7.4 and again centrifuged at 12,000 rpm for 2 min. For further resuspension of the pellet, 100 µl of sterile distilled water was used. Later, the cell suspension was boiled for 10 min, snapped cooled in ice followed by re-centrifugation at 13,400 rpm for 3 min. An aliquot (80 µl) of the resulting supernatant which contained the crude DNA was then transferred to a fresh sterile microfuge tube and stored at -20 ^{0}C until further use. For IAC, the plasmid (pQE-30) DNA was extracted by using Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan) according to the instruction of the manufacturer [21].

Optimization of PCR assay

Previously published species-specific primers (forward: 5'-GCGATTGATGGTGATACGGT-3'; reversed: 5'-AGCCAAGCCTTGACGAACTAAAGC-3') were used to identify the *S. aureus* species which amplified 270 bp PCR product from thermonuclease (*nuc*) gene [22]. A pair of primers (amplifying 187 bp fragment) targeting plasmid pQE-30, which usually utilized to express N-terminally 6xHis-tagged proteins were used as IAC [23]. The *S. aureus* and IAC primer sets were optimized separately as simplex PCR assay and finally optimization of duplex PCR was performed using both primer pairs in a single reaction tube. For performing the simplex PCR, a 25 µl reaction mixture was prepared comprising of 5 µl of 5X GoTaq Flexi Buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM deoxynucleoside triphosphate (dNTP) mix, 1.0 µl of 10 µM of each primer, 0.125 µl of 5U/ µl of GoTaq Flexi DNA Polymerase and 1.5 µl of DNA template (40 ng/uL). In the duplex PCR assay, 1.0 µl of 10 µM of *S. aureus* and IAC primers were added in a single reaction tube with other PCR components in same concentrations as in the simplex one. A negative control was made by replacing template DNA with deionized water. DNA was extracted from the laboratory confirmed *S. aureus* strain and this was used as a positive control while plasmid (pQE-30) DNA was used for IAC. In the initial denaturation step the reaction mixtures were heated at 94 ˚C for 4 min followed by 35 cycles of final denaturation at 94 ˚C for 1 min, annealing of primers at 60 ˚C for 1 min, elongation at 72 ˚C for 1 min and the final elongation at 72 ˚C for 5 min. The amplified products were separated by electrophoresis at 120 V for 60 min with the use of 2% agarose gel (in 1X Trisborate-EDTA buffer) stained with Florosafe DNA stain (First Base Laboratories, Selangor, Malaysia). A gel documentation system (AlphaImager HP, Alpha Innotech Corp., California, USA) was used to visualize the gel. As the molecular size marker, a 50 bp DNA ladder (Promega, USA) was used.

MPN analysis

The MPN values (MPN/g) of viable *S. aureus* in the canned RTE fish samples were calculated following the computer-assisted Microbiological Methods & Bacteriological Analytical Manual (BAM) [24]. The number of positive PCR tubes was entered the BAM Excel spreadsheet and subsequent analysis was done to calculate the bacterial concentration (MPN/g).

DNA sequencing

PCR products were sent to a commercial company (First Base Laboratories, Selangor, Malaysia) for purification and sequencing. The BigDye® Terminator v3.1 cycle sequencing kit in an ABI PRISM 96-capillary 3730xl Genetic Analyzer (Applied Biosystems, USA) was used for sequencing the amplified PCR products in both direction (5'-3' and 3'-5'). The derived sequences were then compared with GenBank sequences using the nucleotide basic local alignment search tool (BLAST) to evaluate any species match and were also aligned with specific gene sequence using MEGA6 software [25] to determine the similarity with specific species.

Results and Discussion

PCR optimization

Initially, simplex PCR of *S. aureus* and IAC was performed using extracted DNA followed by duplex PCR of them to eliminate the probability of potential primer dimer or multimers formation (Figure 1) [26]. In optimization stage of the PCR assays, modification was done in denaturation and primer annealing time for minimizing the occurrence of nonspecific bands. The electrophoretically separated amplified PCR products were visualized in gel documentation system. The simplex assay of *S. aureus* amplified 270 bp PCR product from *nuc* gene (Figure 1, lane C) whereas IAC was a target of pQE-30 plasmid which amplified 187 bp fragment (Figure 1, lane B). The duplex PCR system of *S. aureus* and IAC amplified both 270 bp and 187 bp products respectively, in a single reaction (Figure 1, lane D). The IAC was incorporated in all PCR assays to rule out false negative results. DNA sequence analysis of the amplicon generated by the referenced *S. aureus* strain showed 99% similarity to the NCBI database (Accession no: CP034441.1), thus validating the amplified PCR product.

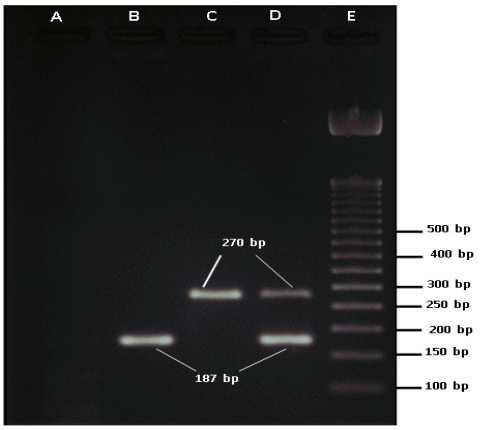

Figure 1: The gel image of duplex PCR assay of *S. aureus* targeting 270 bp site of *nuc* gene and IAC (Internal Amplification Control). In the gel image: *Lane* A represents negative control; *Lane* B, PCR products of IAC; *Lane* C, PCR products of *S. aureus*; *Lane* D, PCR products of *S. aureus* and IAC*; Lane* E, DNA ladder.

Enumeration of S. aureus in canned fish

Canning is the process which is intended to destroy the deleterious microorganisms in RTE food. However, due to improper handling, processing and inadequate cooling, cans often become the potential sources of microbes [27]. In this study, 32 RTE canned fish samples were positive for *S. aureus* out of 62 samples. The occurrence of the target bacteria was 51.61% with a microbial load of 9.50 $x10^{-1}$ MPN/g - 4.41 x 10^{3} MPN/g and this level is mentioned as the borderline (20 - ≤ 10^{4}) according to Health Protection Agency Guideline (UK) (2009). The bacterial contamination may be due to poor handling, processing and temperature mismanagement through the process. Seven (11.6%) samples had a microbial load of 3 to <100 MPN/g and another seven samples had a microbial load within 100 to <1000 MPN/g and the rest of the samples had a higher microbial content of >1000 MPN/g. However, pathogenic bacteria are often unevenly distributed in foods and thus the contamination levels that are usually observed, and accordingly the subsequent interpretation made based on this, may be varying between sub-samples. Since low numbers of these organisms are often detected in ready-to-eat foods which are believed to be related to food poisoning outbreaks and these foods are also consumed by a large number of people of vulnerable groups, further investigation by the enforcement authority in association with the Food Examiner is necessary to make a conclusive decision. In general, the people of vulnerable group are more susceptible to these infections with a higher risk to develop more serious disease.

Our study concerns the identification, quantification and the occurrence of *S. aureus* using MPN-PCR method that offers higher sensitivity than conventional PCR alone which was also shown in previous study [28]. Moreover, the MPN-PCR can quantify the target bacteria and detect the viable but not culturable bacterial cells.

Previously, Saito et al. [29] reported a higher contamination rate of processed fish (26.6%) by *S. aureus* as compared to that of unprocessed fish (14.3%) in Japan which indicates that the fish could have been contaminated with *S. aureus* from humans involved in processing and handling. It was also mentioned that the number of staphylococcal food poisoning incidences in Japan had been reduced in tandem through the improvement in the processes of food production, distribution and handling and also due to increased awareness regarding food sanitation. Recently, 26% of sushi and sashimi sold in Malaysia have been reported to be contaminated with *S. aureus* [15] while another study revealed contamination in 53% of tested RTE food samples [30]. A total of 267 *S. aureus* strains were isolated from 244 RTE foods in Switzerland [31] and 21.23% RTE foods including milk-based product marketed in Hanoi, Vietnam were also found to be contaminated with *S. aureus* [32]. In Korea, *S. aureus* contamination was found in 8.6% of RTE food products [33]. There are a number of previous reports

that studied low risk fish and fishery products including fresh fish [34], frozen products [15] and dried or salted fish products [35], but scanty study is available on the incidence of *S. aureus* in RTE canned fish.

Conclusion

In conclusion, determining the occurrence and quantification of *S. aureus* in RTE canned fish may help to promote the awareness toward RTE canned food and to improve current practices of manufacturing, canning and post-canning processes in Malaysia. The optimized MPN- duplex PCR (*S. aureus* and IAC) method can facilitate routine screening and quantification of *S. aureus* at any stage in food supply chain. The findings of this study suggest that handling and processing should be carried out with especial caution in order to maintain the microbial quality of RTE food. Further investigations regarding virulence characteristics of the isolates such as ability for enterotoxin production, antimicrobial resistance, and biofilm formation along with identifying possible contamination sources might be performed at a large scale for better evaluation.

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Conflict of interest

All authors declare that there is no conflict of interest to publish the content of this article.

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