Real-time PCR application in confirmation test of *Salmonella Typhimurium* on instant noodle

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ARTICLE INFO

DOI:10.46223/HCMCOUJS.tech.en.13.1.2479.2023

Received: September 24th, 2022

Revised: January 09th, 2023

Accepted: January 11th, 2023

Keywords:

bacteria; DNA; PCR; salmonella; testing

ABSTRACT

Real-Time PCR is a molecular biology testing instrument used for various types of food product testing applications to monitor the quality of a product. In addition, the real-time PCR technique can also be used to test the detection of pathogenic bacteria in some foods and processed products. The purpose of this study was to detect Salmonella typhimurium using real-time PCR. This research can be a source of information and a reference for similar research. The test method used is the real-time PCR method with the green SYBR kit. Data analysis was carried out by looking at the results of interpreting Ct values and Tm values. Positive results are indicated by the formation of the Ct curve and the Tm curve. From the research conducted, it was found that the Ct value was in the range of 11.90 - 12.10 with an average of 12.02, while the Tm value was in the range of 85.10 - 85.20 with an average of 85.15. From the study results, it can be concluded that the use of real-time PCR for testing the detection of Salmonella typhimurium in instant noodle products can be applied.

1. Introduction

Real-Time PCR is a molecular biology testing instrument that is used for various types of food product testing applications to monitor the quality of a product. In addition, the real-time PCR technique can also be used to test the detection of pathogenic bacteria in several foods and processed products. The application of real-time PCR in the development of molecular-based testing causes the testing time used to perform the detection test to be slightly shorter when compared to using conventional testing methods (Ding et al., 2017). These developments gave birth to various studies that compared several *Salmonella* testing techniques between conventional, rapid tests and real-time PCR (Ibrahim, El-Ghany, Nasef, & Hatem, 2014; Mata & Vanetti, 2012; Paniel & Noguer, 2019; Siala et al., 2017).

Several studies that have applied real-time PCR in testing *Salmonella spp*. have been carried out on several products, including food (Zhao et al., 2021), meatballs (Sophian, Purwaningsih, Muindar, Igirisa, & Amirullah, 2021a; Sophian, Purwaningsih, Muindar, Igirisa, & Amirullah, 2021b), traditional medicines (Sophian, Purwaningsih, Muindar, et al., 2021a), probiotic products (Greve-Peterson & Legan, n.d.) and health supplements (Sophian, Purwaningsih, Lukita, & Ningsih, 2020). The use of real-time PCR instruments provides relatively faster test results when compared to conventional methods, which require multiple incubation processes to obtain test results. Cutting the testing time in conducting the detection of pathogenic

bacteria in monitoring the quality of a product is a positive step that can be taken, where the faster the test results, the faster conclusions will be drawn. The development of several *Salmonella* identification test methods has been validated to test the robustness of the method so that it can be used as a valid test method that can be responsible for the quality of the test results (Carlin et al., 2020; Sturza et al., 2021).

So based on this background, this research was conducted to see how the real-time PCR application technique in detecting the presence of *Salmonella typhimurium* in instant noodle products so that this research can be a source of information and reference for similar research. Besides that, this research is expected to be a source of information that can be considered in developing a validated method for testing the safety of food products.

2. Methodology

2.1. Materials

The ingredients in this study were Instant Noodles, Buffered Peptone Water (BPW) Oxoid catalog number CM0505, Tryptic Soy Agar (TSA) MERCK catalog number 1.05458.0500, Tris HCl 0.1%, NaOH 50 Molal, and QuantiNova SYBR Green (Qiagen) PCR Kit.

2.2. Sample

Instant noodles consisting of 10 packages weighed 10 grams. They added 90mL of Buffered Peptone Water (BPW), spiked with 1mL of Salmonella typhimurium positive culture, then homogenized and incubated in an incubator at 35 - 37°C for 24 hours. After that, take 01 ose and scratch it on TSA media slanting agar and incubate at 35 - 37°C for 24 hours. The results of the incubation were taken 01 ose then cloudy in a test tube containing 5mL of NaCl to form a culture solution with turbidity suspension equal to 1 McFarland. Save the suspension for use as a DNA template.

2.3. Master mix setup

The test master mix was made with a total volume of 20µL consisting of 10µL Sybr green master mix, 1µL each forward primer and reverse primer, 3µL nucleotide-free water, and 5µL template DNA (Sapiun et al., 2020; Sophian, Purwaningsih, Muindar, et al., 2021b).

2.4. PCR setup

Rotor Gene Q (QIAGEN 5 Plex) was used for real-time PCR analysis. The tool uses the following method: Denaturation 95°C for 45 seconds and Annealing/Extension 60°C for 45 seconds. The primer sequences are InvA Forward (5'-ATC AGT ACC AGT CGT CTT ATC TTG AT-3'), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3').

2.5. Control

The controls used in this study consisted of positive controls and negative controls. These two controls are useful to help interpret the results when concluding research data (Sapiun et al., 2020; Sophian, Purwaningsih, Igirisa, et al., 2021).

2.6. Data analysis

Data analysis was carried out by looking at the interpretation results based on the Ct and Tm values. Positive results are indicated by the formation of the Ct curve and the Tm curve (Sophian, Purwaningsih, Muindar, et al., 2021a; Sophian, Purwaningsih, Muindar, et al., 2021b).

3. Result and discussion

3.1. Enrichment results on Enrichment Broth Media (BPW)

The enrichment stage was carried out using BPW media, the spkied sample was then incubated for 24 hours at 35 - 37°C, and the results of the observations were presented in Table 1 below.

Table 1

Observation result

No.	***Enrichmen Media***	***Result***
1.	Buffered Peptone Water	Cloudy
2.	Buffered Peptone Water	Cloudy
3.	Buffered Peptone Water	Cloudy
4.	Buffered Peptone Water	Cloudy
5.	Buffered Peptone Water	Cloudy
6.	Buffered Peptone Water	Cloudy
7.	Buffered Peptone Water	Cloudy
8.	Buffered Peptone Water	Cloudy
9.	Buffered Peptone Water	Cloudy
10.	Buffered Peptone Water	Cloudy

From Table 1 above, it can be seen that all pre-enrichment results on BPW enrichment media showed cloudy results in all samples, which means that all spiked target bacteria in 10 instant noodle samples experienced enrichment or growth. This is in line with the opinion expressed by Sophian, Purwaningsih, Muindar, et al. (2021b), which states that the success of the pre-enrichment process on BPW media can be seen from the occurrence of turbidity in the culture of the test sample.

3.2. Amplification results

Real-time PCR analysis was carried out using a qualitative test method using SYBR green; the results obtained were in the range of 11.90 - 12.10 with an average of 12.02, while the value of Tm was in the range of 85.10 - 85.20 with an average of 85.15. Complete data is presented in Table 1.

Table 2

Amplification results

Sample	***Analysis Value***	
	Ct	***Tm***
1	11.90	85.10
2	12.10	85.20
3	12.10	85.20
4	12.10	85.10

Sample	***Analysis Value***	
	Ct	***Tm***
5	12.10	85.10
6	11.90	85.10
7	11.90	85.20
8	11.90	85.20
9	12.10	85.20
10	12.10	85.10
Average	12.02	85.15

The results of the analysis showed the formation of a sigmoid Ct curve for cycle analysis and a melt curve for Tm analysis. The amplification result curve is shown in Figure (1) below.

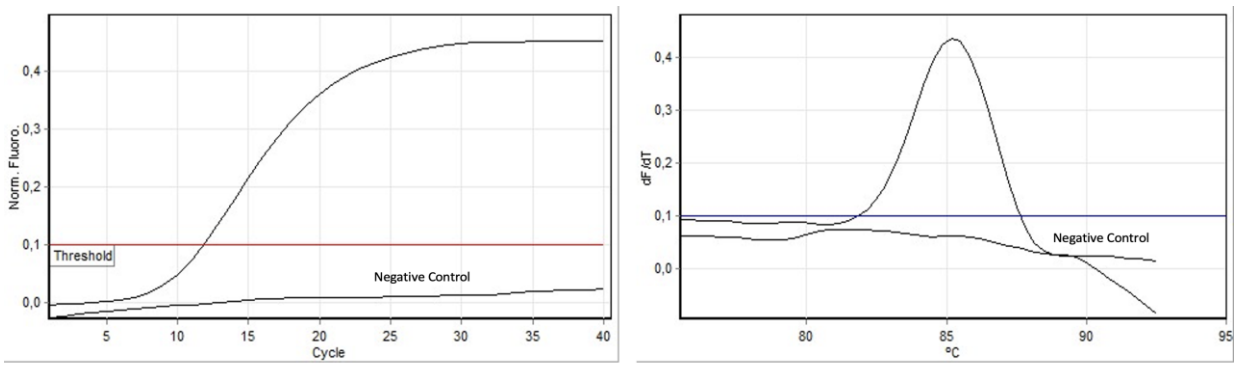

Figure 1. Results of analysis. (a) Ct Value; (b) Tm Value

In Cycling analysis, the value of Ct is influenced by the concentration value of the template DNA used, while for melt curve analysis, the value of Tm is strongly influenced by the composition of nucleotide bases. The Tm value is formed when the fluorescent signal gives information when the DNA bands begin to separate after the annealing process. The melt curve that is formed will produce a curve with a specific single peak from each detected band. However, in every melt curve analysis, sometimes double peaks can also occur. However, the presence of double peaks does not mean that it can be used as a justification that the test results used are not specific (Dwight, Palais, & Wittwer, 2011).

3.3. Discussion

In this study, the sample used was instant noodles. This sample selection is because one of the quality test parameters on instant noodle products is the detection test for *Salmonella spp*. The initial stage of testing starts from pre-enrichment by using BPW enrichment media to grow the target bacteria to be detected. From the results of monitoring the BPW media, all samples showed a change in the colour of the liquid from brownish and slightly clear to cloudy after being incubated for 24 hours at a temperature of 35 - 37°C. This colour change indicates the presence of bacterial growth in the sample being tested. This is in line with the opinion expressed by Sophian et al. (2020) which states that a successful enrichment process will be marked by changes in the BPW media becoming cloudy.

The DNA isolation technique used in this study is the direct PCR technique, which is the technique of using templates directly without going through the lysis process to isolate DNA. The advantage of this technique is that the test time is faster and cheaper because it does not require an extraction kit. According to Sophian, Purwaningsih, Muindar, et al. (2021b), the direct PCR technique causes the DNA templates used in the testing process to tend to have high inhibitors. In the direct PCR technique, the lysis process is expected to occur perfectly so that the target DNA will be amplified. The initial denaturation process that uses high temperatures will break the cell wall so that the rupture of the cell wall will release the DNA in the cell for amplification.

When carrying out the test, the matrix character of the sample being tested will affect the testing technique, where the physical, chemical, or biological characteristics of the sample to be used will also affect the success of the test (Giacomazzi, Leroi, & Joffraud, 2005; Wilson, 1997). Testing techniques using molecular test principles have advantages in several ways when compared to conventional test methods, including the relatively faster test time obtained (Jobling & Gill, 2004; Lockley & Bardsley, 2000; Tang, Procop, & Persing, 1997). According to Postollec, Falentin, Pavan, Combrisson, and Sohier (2011), the identification of species by molecular techniques using real-time PCR (qPCR) is a fast and reliable method. Two methods are often used in conducting analyzes to detect DNA of species using real-time PCR, namely the TaqMan and SYBR Green methods (Beutin, Jahn, & Fach, 2009; Fukushima et al., 2009). This is in line with Sophian et al. (2020) who states that the use of the SYBR Green method is an effective and easy method to detect Salmonella.

The gene used in this study is the invA gene which was chosen because the invA gene is a specific gene for identifying *Salmonella spp*. The gene is located in the area of *Salmonella* pathogenicity island (SPI-R) which has an operon that functions as a repository of genetic information. The InvA gene can be detected quickly using a PCR method that has optimized its components (Sophian, Purwaningsih, Muindar, et al., 2021b). The use of the invA gene for the detection of *Salmonella spp.* using real-time PCR has been developed and validated by Malorny, Hoofar, Bunge, and Helmuth (2003) to see the performance of the primers used. Validation is an important step in developing a standard method to produce a good test method. With validation, the reliability of the test method used can be guaranteed.

4. Conclusions

The results showed that the use of the real-time PCR method in the Salmonella typhimurium detection test on instant noodle products can be applied, this is evidenced by the detection of target bacteria during testing.

5. Conflict of interest

The team of authors declares that there is no conflict of interest, and no affiliation or connection to or with any entity or organization, which could raise questions about bias in the discussion and conclusions of the research paper.

ACKNOWLEDGEMENTS

The authors thank the Head of the National Food and Drug Testing Development Center for policy support on the use of laboratories in this study.

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