Research Article

Detection of *Salmonella typhimurium* ATCC 14028 in Powder Prepared Traditional Medicines Using Real-Time PCR

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Abstract

The detection of *Salmonella typhimurium* ATCC 14028 using real-time PCR on powdered traditional medicinal products was carried out in the microbiology and molecular biology testing laboratory of the Food and Drug Administration in Gorontalo. This research aims to provide a reference for alternative testing methods in testing the products of traditional powder preparations on the market. The sample consisted of 10 traditional powder preparations spiked with positive control of *S. typhimurium* ATCC 14028 phase 2. The method used in the study was real-time PCR analysis using the SYBR® Green method, while DNA isolation using the direct PCR method. Data analysis was performed by analyzing the sample's melting temperature (Tm) curve and comparing it with positive control. The results showed that *S. typhimurium* ATCC 14028 was detected in samples at an average Tm value of 84.18°C, with ranges of 84.0-84.5°C. For positive control, the Tm value was at 85.2°C, while for the negative control, the Tm value was not detected. Based on these data, it can be concluded that *S. typhimurium* ATCC 14028 in traditional medicine products powder preparations can be detected using real-time PCR.

INTRODUCTION

Traditional medicines are ingredients in the form of the plant, animal, mineral substances, galenic preparations, or mixtures of these ingredients, which have been used for treatment from generation to generation and can be applied following the prevailing norms in society[1,2]. Traditional medicinal preparations consist of various types, including powder, instant powder, pills, caplets, capsules, and other simple forms. The simple manufacturing process sometimes raises the possibility of contamination by pathogenic bacteria in these traditional medicinal preparations[3,4].

So far, the method of detecting pathogenic bacteria in traditional medicinal products is still using conventional test method guidelines using selective media. However, the development of science and technology has led to several other alternatives in detecting pathogenic bacteria, one of which is using molecular techniques[5,6]. Molecular analysis to identify pathogenic bacteria using real-time has advantages when compared to

conventional methods[7]. The average time required to identify pathogenic bacteria by molecular techniques is faster when compared to conventional methods[8].

Regarding the quality requirements of traditional medicines, contaminants from pathogenic bacteria, including Salmonella, Shigella, and Clostridium, should not be found[9]. Members of the genus Salmonella can cause fever and digestive tract diseases, such as *S. typhimurium*[10]. Among several biomolecular techniques for Salmonella detection, polymerase chain reaction (PCR) is the most frequently used. Real-time PCR analysis has better sensitivity in detecting the DNA of Salmonella[11].

One of the traditional medicines that are often found is in the form of powder preparations. Traditional medicine powder preparations are homogeneous granules with an appropriate, acceptable degree, made from simplicia or a mixture with extracts brewed with hot water[12]. These preparation forms can contain microbial contamination such as *S. typhimurium*, especially if processed and stored not cleanly and hygienically[3]. Therefore, this research aims to provide an alternative testing method for *S. typhimurium* contamination in traditional powder preparations on the market. The novelty aspect discussed in this study is to provide a source of reference information in the test for detecting pathogenic bacteria in traditional powdered products using real-time PCR. This research is a follow-up study because the data obtained will be used to evaluate several different types of samples so that the effectiveness of the testing methods and techniques used on the various types of samples used can be evaluated.

MATERIALS AND METHODS

Materials

The materials used in this study were samples of traditional medicine powder, tryptic soy broth (TSB) and agar (TSA) enrichment media, xylose lysine deoxycholate (XLD) and brilliant green agar (BGA) selective media, and a PCR kit with QuantiNova SYBR® Green (Qiagen).

Methods

Sample criteria and sampling period

The main criteria for the sample used in this study was a traditional medicine powder consisting of several traditional medicines for shooting pain or "pegal linu" and herbal medicine for women's health. Sampling was carried out at markets and pharmacies in Gorontalo City from October to December 2020.

Sample preparation

The sample consisted of 10 types of traditional medicinal powder preparations spiked with positive control *S. Typhimurium* ATCC 14028 phase 2.

Isolation on selective media

A total of 10 g of sample was weighed and then added 90 mL of TSB and incubated at 35-37°C for 18-24 hours. One ose of each incubated sample was then taken and streaked on BGA and XLD selective media, then incubated at 35-37°C for 18-24 hours. The observations of colonies growing on selective media were then recorded.

DNA isolation

The results of isolation from selective media are then enriched on media so that it is tilted. The enrichment result was then diluted in physiological NaCl to obtain turbidity equivalent to the McFarland standard number 1. This equalization result was then used as a DNA template[13].

Real-time PCR analysis

Melt curve analysis was carried out using real-time PCR (QIAGEN 5-plex) with the two-step cycling method: denaturation 95°C for 45 seconds and annealing/extension 60°C for 45 seconds. The primers used to detect *S. typhimurium* were using invA forward

primers (5'-ATC AGT ACC AGT CGT CTT ATC TTG AT-3 '), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3').

Reaction setup

The total volume of the master mix was 10 μL, consisting of 5 μL SYBR® Green master mix, 1 μL forward primer, 1 μL reverse primer, 1 μL water-free RNase, and 2 μL template DNA[14].

Positive control

The positive control used was *S. typhimurium* ATCC 14028 phase 2, enriched and scratched by the media to make it tilted. The enrichment result was then diluted in physiological NaCl to obtain turbidity equivalent to the McFarland standard number 1[14].

Negative control

The negative control used was no template control (NTC), a master mix combined with primer and nucleic acid-free water. The total negative control volume was 10 μL, consisting of 1 μL forward primer, 1 μL reverse primer, 3 μL RNase free water, with the rest was SYBR® Green master mix[14].

Specificity

Specificity was carried out by replacing the target bacterial DNA with other bacterial DNA to see whether the non-target DNA was amplified or not. In addition, another function of the specificity test was to examine whether the primer used was specific or not.

Limit of detection

The limit of detection (LOD) was carried out by replacing the sample DNA template with a positive control diluted 10 times. This step aims to determine the sensitivity of the tool in detecting low concentrations of target DNA.

Data analysis

Data analysis was carried out by seeing the amplification results of the melting temperature (Tm) value, the melting point at the temperature at which the melt

occurred, and comparing the melting point for the positive control[14].

RESULTS AND DISCUSSION

Isolation on selective media

The isolation process on selective media began with enrichment on TSB media. In this medium, the target bacteria could not be distinguished from other bacteria that could have grown. The general characteristic was only a change in the color of the enrichment medium from clear brownish-yellow to brownish-yellow cloudy[15]. The enrichment stage was carried out to prevent quantifying diseased cells that conventional methods could not be grown/counted[16].

Isolation on selective media was using BGA and XLD, the specific media for isolating Salmonella pathogenic bacteria. The profile of Salmonella on XLD was a colony that is round in shape. In BGA, the colony was white to translucent, whereas, in XLD, it could be colorless, very bright, slightly shiny, and transparent (medium color) with a black center, surrounded by a pink area and a yellow border, or a pink to reddish color, with a black or colorless buffer core[17,18].

Brilliant green agar comprises peptone, tryptone, yeast extract, lactose, sucrose, sodium chloride, phenol red, brilliant green, and agar. Each of the constituent components of the BGA has its respective functions, including as a source of nutrition for amino acid or polypeptide raw materials, maintaining the osmotic balance of the media, sources of carbohydrates that will be needed in the fermentation process, as well as an acid-base indicator. While in XLD media, Salmonella has a translucent round shape with a black spot in the middle. The color change is caused by the fermentation of glucose by Salmonella into organic acids such as lactic, acetic, and formic acids, resulting in a decrease in pH[19]. Xylose lysine deoxycholate is a non-autoclave medium whose

components will be damaged if harvested at temperatures above 100°C. Xylose carbohydrates in XLD play a role in the fermentation process to condition the pH of the media to become acidic, thereby increasing the pH to become alkaline. Meanwhile, decarboxylate enzymes produce amines or diamines and carbon dioxide by breaking down amino acid groups. Xylose lysine deoxycholate media is a selective medium for *S. typhimurium* because it has sodium deoxycholate, an inhibitor for Gram-positive bacteria[18].

Real-time PCR analysis

Real-time PCR analysis was performed using a qualitative test method using SYBR® Green, and the results were presented in **Table I**. The data were obtained from 10 samples which were 10 types of traditional medicine with the powder preparation form. The Tm value from real-time PCR analysis was 84.0-84.5°C, with an average of 84.18°C. This value is not much different from the positive control at the Tm value of 85.2°C. This difference could be caused by several things, including the concentration of the template DNA used was not the same. This difference in concentration occurred because the template DNA was not isolated using conventional isolation techniques but instead used the direct PCR method[20].

Table I. Real-time PCR data analysis

Samples	Tm Value (°C)	Negative Control	Specificity	LOD	Positive Control
1	84.0	-	-	85.0	85.2
2	84.5				
3	84.3				
4	84.3				
5	84.0				
6	84.0				
7	84.0				
8	84.2				
9	84.0				
10	84.5				
Average	84.18	-	-	85.0	85.2

The results of real-time PCR amplification using the qualitative analysis method SYBR® Green were shown

in **Figure 1**. It could be seen that the positive control shown by the red line indicates an amplification at the Tm 85.2°C, while for the negative control shown with the black line, there was no amplification. The samples shown in green lines indicate an amplification similar to the positive control; this indicates that *S. typhimurium* was detected from the samples.
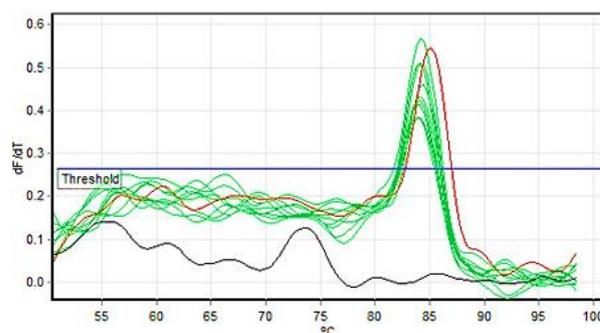

Figure 1. Melt curve analysis for Tm. Green: samples; Red: positive control; Black: negative control

In **Figure 1**, melt curve analysis shows that the peak of the curve point varies slightly. This variation is due to the composition and size of the nucleotides[21]. In SYBR® Green analysis, a fluorescent signal will emit when the DNA bands separate during the annealing stage. A single peak detected in the melting process indicates the specific level of the detected band. If there is a band difference in the single peak in the melt curve analysis, it can predict the presence of mutations in the detected species[22].

Molecular testing techniques have their challenges, in which DNA extraction techniques such as physical, chemical, or biological characteristics of the sample to be used will also affect the test's success[23]. Compared with conventional techniques, molecular testing techniques have advantages in several ways, including the relatively faster testing time obtained. Species identification using real-time PCR is a fast and reliable alternative method in developing the molecular world[24]. At least two real-time PCR methods are often used in analyzing species DNA: the TaqMan® and SYBR® Green methods[25].

CONCLUSION

The detection method of *S. typhimurium* in powder preparations of traditional medicinal products using real-time PCR with the SYBR® Green method can be used, as indicated by the results of all samples tested showing noticeable results.

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AUTHORS' CONTRIBUTION

Alfi Sophian: conceptualization, data curation, data analysis, writing-original draft & editing. **Ratna Purwaningsih**: writing-review & editing. **Muindar**: writing-review & editing. **Eka Putri Juniarti Igirisa**: writing-review & editing. **Muhammad Luthfi Amirullah**: writing-review & editing.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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