Detection of *Salmonella typhimurium* ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR)

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Abstract. Sophian A, Purwaningsih R, Lukita BL, Ningsih EC. 2018. Detection of *Salmonella typhimurium* ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR). Biofarmasi J Nat Prod Biochem 18: 61–65. Detection of *Salmonella typhimurium* ATCC 14028 using Real-Time PCR (qPCR) on health supplement products was carried out in the microbiology and molecular biology testing laboratory of the Food and Drug Supervisory Office in Gorontalo. The purpose of this study was to provide an alternative testing method reference in the testing of liquid supplement health supplement products in the market. The sample consisted of 35 samples of liquid supplement health supplements spike with positive control of *Salmonella typhimurium* ATCC 14028 phase 2. The method used in the study was qPCR analysis using the SYBR Green method, whereas DNA isolation using the direct PCR method. Data analysis was performed based on 2 main criteria: (i) Ct (Cycle threshold) analysis, which is looking at the value of the sample Ct and comparing it with controls and (ii) analysis of melting temperature (Tm), which is the melting point at the temperature at which melt occurs and comparing the melting point to the positive control. The results showed that in the sample, *Salmonella typhimurium* ATCC 14028 was detected at an average Ct value of 14.43, and an average Tm value of 86.05, for the specificity, LOD and positive control tests were all amplified. For negative controls, Ct and Tm values were not detected. Based on these data it can be concluded that real-time PCR (qPCR) can be used to detect *Salmonella typhimurium* ATCC 14028 in liquid supplement health supplement products.

Keywords: Health supplement, phase, qPCR, *Salmonella typhimurium*, SYBR green

Abbreviations: HEA: Hekton Enteric Agar, LOD: Limit of detection, TSA: Tryptic Soy Agar, TSB: Tryptic Soy Broth, XLD: Xylose Lysine Desoxycholate Agar

INTRODUCTION

Health supplements are products intended to fulfill nutritional needs, maintain, enhance or improve health functions, have nutritional value or physiological effects, contain one or more ingredients in the form of vitamins, minerals, amino acids and other non-plant ingredients which can be combined with plants. Health supplements can be made in the form of tablets, pills, capsules, oral liquids, powders, and granules or gummy. Health supplements must be made by using safe, useful, and quality raw materials by the provisions of the Indonesian Pharmacopoeia, Indonesian Herbal Pharmacopoeia, other countries pharmacopoeia or recognized scientific references (NADFC 2019). To support the safety of the quality of health supplement products available in the market, it is necessary to have the latest test methods developed according to the advancement of science and technology. One such latest method is the use of molecular analysis in the detection of pathogenic bacterial contamination in health supplements.

Molecular analysis to identify pathogenic bacteria using real-time PCR has advantages over conventional methods. The average time required if the identification of pathogenic bacteria is done conventionally is about 3-5 days, whereas, it only takes 50-52 hours if PCR method is used as isolation technique (24 hours for enrichment samples, 24 hours for selective enrichment, and 1.5 hours for real-time PCR analysis).

One of the quality check parameters of health supplement products is that it should be free from pathogenic *Salmonella* bacteria: therefore the mechanism of *Salmonella* identification is becoming increasingly necessary. Among the several molecular techniques used in the detection of *Salmonella* most commonly used is the polymerase chain reaction (PCR). Real-time PCR analysis (qPCR), which shows high sensitivity and reproducibility in the amplification of certain DNA fragments and can measure the presence of *Salmonella* DNA (Oliveira et al. 2018).

In recent years, several studies on the detection of *Salmonella* in various samples such as meat and poultry (Cataramo et al. 2006); raw pork sausages (Wang et al. 2004); pasteurized milk, ground beef, and sprouts (Mecanoglu et al. 2005); water and milk (Jothikumar et al. 2003); samples of prepared foods, raw meat, and poultry (Cheung et al. 2004); minced beef, fish and raw milk (Perelle et al. 2004); raw and ready-to-eat beef products (Ellingson et al. 2004); and chicken intestines, cloacal
swabs, chicken carcasses (Eyigor et al. 2003; Eyigor et al. 2002) have been carried out.

According to Brooks et al. (2007), Salmonella can cause enteric fever, which is caused by Salmonella typhi called Typhoid fever, Septicemia, a fever caused by Salmonella choleraesuis, and Gastroenteritis, a digestive tract disease caused by food poisoning that contains Salmonella typhimurium.

NADFC Regulation No. 17 of 2019 concerning Quality Requirements for Health supplements, regulates the limits of contamination of pathogenic bacteria in health supplements, where herbal-based health supplements may not contain pathogenic bacteria like Salmonella spp. Health supplement quality requirements must be applied before and during health, supplements are in circulation. Therefore monitoring of the product is very important to ensure that the circulating products are free from pathogenic bacterial contaminants. The quality requirements for health supplements must comply with the provisions of the Indonesian Pharmacopoeia and Indonesian Herbal Pharmacopoeia. Based on this, the research was conducted to develop alternative methods in the detection of Salmonella in liquid supplement health supplement products.

MATERIALS AND METHODS

Materials

The sample materials used in this study were liquid health supplements, distilled water, TSB enrichment media, TSA, XLD, and HEA selective media, PCR kit uses QuantiNova SYBR Green (Qiagen).

Sample setup

Total 35 samples of liquid supplement health supplements spike with positive control of Salmonella typhimurium ATCC 14028 phase 2 were used during the present study.

Isolation in selective media

For isolation, 10 grams sample for solid samples were weighed out while it was pipette out 10 ml for a liquid sample. To this 90 mL TSB was added and then incubated at 35-37°C for 18-24 hrs. After incubation, one sample was scratch on the HEA and XLD selective media and then incubated at 35-37°C for 18-24 hrs. Observations of colonies growing on selective media were recorded at regular intervals.

DNA isolation

DNA isolation not carried out because the direct PCR method was used. The results of isolation from selective media were then enriched on the media to be skewed. The results of enrichment were then stabilized in physiological NaCl and equalized to standard 1 MacFarland. The results of this equalization were then be used as DNA templates.

qPCR analysis

Cycling and melt curve analysis were carried out using qPCR (QIAGEN 5 Flex) with the 2 step cycling method: Denaturation 95°C for 45 sec and Annealing/Extension 60°C for 45 sec. The primer used to detect Salmonella typhimurium using InvA Forward primers (5'-ATC AGT ACC AGT 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 DNA template.

Positive control

The positive control used was Salmonella typhimurium ATCC 14028 phase 2 which was enriched and etched on the media to make it tilt. Salmonella typhimurium ATCC 14028 colony was then dredged in physiological NaCl and equalized to standard 1 MacFarland.

Negative control

The negative control was NTC (No Template Control), a master mix combined with primers and free water from nucleic acids. The total negative control volume was 10 µL consisting of; master mix Sybr green, 1 µL forward primer, 1 µL reverse primer, 3 µL RNase free water

Specificity

Specificity testing was done by mixing the Salmonella typhimurium ATCC 14028 colony with the colony of Escherichia coli ATCC 25922 and Listeria monocytogenes ATCC 7644 in a ratio of 1: 1: 1 using the reference standard 1 MacFarland.

LOD

For LOD analysis, it was done by adding a 10: 1 sample DNA template from a positive DNA control template. In the positive control, the DNA template used was 2 µL, then the DNA template for LOD is 0.2 µL.

Data analysis

Data analysis was carried out based on 2 main criteria which included: (i) Ct (Cycle threshold) analysis which looked at the value of sample Ct and compared it with controls. (ii) Analysis of melting temperature (Tm), which is the melting point at the temperature at which melt occurs and comparing the melting point to the positive control.

RESULTS AND DISCUSSION

Isolation in selective media

The results of observations on HEA and XLD selective media can be seen in Figure 1. Based on the results of isolation on HEA selective media, the colony was grey to black in the middle, while on XLD media, it was translucent to a black in the center.
The choice of HEA and XLD selective media is because these two selective media are specific media for isolation of Salmonella pathogenic bacteria. Andrews et al. (2007) explained that the profile of Salmonella on HEA and XLD media has a similar shape, which is the presence of black spots or not in the middle of the colony. A different matter was stated by Murray et al. (1999) which states that the HEA and XLD media are often used together in the detection of Salmonella because both of these media can also isolate Shigella. Both HEA and XLD have H2S indicators that can detect Salmonella from lactose positive indicators.

Real-time PCR analysis

PCR real-time analysis was performed using the qualitative SYBR green method and the results obtained are presented in Table 1.

Based on the analysis of 35 samples, the obtained results showed that all samples detected were positive for Salmonella typhimurium ATCC 14028, as seen from samples compared with positive controls. The detection limit used was 1:10 from the sample template. This comparison was set because there is no definite standard of LOD in the detection of Salmonella typhimurium ATCC 14028, so that it is determined by itself with a dilution system 10 times smaller than the DNA template. The specificity test was made from 2 groups of gram-negative and gram-positive bacteria to see if there was an influence on the ability of the method to detect specific DNA. The Escherichia coli ATCC 25922 were selected because these bacteria belong to the same gram-negative group as Salmonella typhimurium ATCC 14028 and Listeria monocytogenes ATCC 7644 represents a group of gram-positive bacteria.

The results of cycling analysis by looking at the Ct value showed that the qPCR analysis showed Salmonella typhimurium ATCC 14028 in samples detected at Ct 14.43, LOD with a spike of positive control 10 times smaller than the sample turned out to be able to be detected at Ct 14.8, specificity was detected at Ct 19.47 difference. which is quite far from the value of Ct samples and LOD against Ct specificity indicates that the addition of templates from Escherichia coli ATCC 25922 and Listeria monocytogenes ATCC 7644 influence the ability to detect Ct values. Positive control at 15.7 different Ct values can also be caused by several factors including because the PCR direct concentration method and DNA template purity were not carried out in the measurement process, the template concentration between the sample and control was not the same so that the Ct values could be different. For negative controls give unamplified results. This gives information that the master mix or in the test process does not occur contamination of the sample and positive control, as shown in Figure 2.

The results of the melt curve analysis by looking at the Tm value showed that the qPCR analysis showed Salmonella typhimurium in samples detected at Tm 86.05, LOD at Tm 86.0, specificity at Tm 83.8 and positive control at Tm 85.2. While the negative control did not show any Tm value due to not being amputated in the qPCR amplification process, as shown by (Figure 3). As with the Ct analysis, the difference in the value of Tm was also directly proportional to the difference in the value of Ct. This was because of the DNA template used not analyzed for its purity and concentration as for DNA isolation PCR direct technique was used and the concentration of samples, positive control, LOD, and sensitivity are unknown.

Table 1. qPCR data analysis

| qPCR analysis | Treatment | Negative control (NTC) | LOD | Specificity | Positive control |
|---------------|-----------|------------------------|-----|-------------|-----------------|
| Ct values     | 14.43     | 14.8                   | 19.47| 15.7        |                 |
| Tm values     | 86.05     | -                      | 86.00| 83.8        | 85.2            |

Note: Ct and Tm values are the average value of 35 data replications

Figure 1. Salmonella profile on selective media HEA (blue) and XLD (red)

Figure 2. Ct analysis

Figure 3. Melt curve analysis (Tm)
Discussion

Detection of Salmonella in liquid supplement health supplement products using real-time PCR was carried out by a qualitative method using the Quantinova SYBR Green (Qiagen) kit. This study was conducted using a sample of 35 liquid supplement packs on the spike with Salmonella typhimurium ATCC 14028, while for specificity used Escherichia coli ATCC 25922 and Listeria monocytogenes ATCC 7644.

XLD media gave positive results of Salmonella by positive control, namely translucent spherical colonies with black spots in the middle. These results are in line with research conducted by Maddocks et al. (2002) and Nye (2002). According to Braid et al. (1995), the color change is based on observations of the HEA selective media (Figure 1) shows that on selective media, the colonies are greenish-blue with or without black at the center of the colony. In large concentrations or cultures, many colonies could be glossy black or appear to be colonies that are almost entirely black (BAM 2007). HEA is a selective media that does not have the ability to selectivity like XLD. Gaillot et al. (1999) tested 508 Salmonella samples using HEA with 16 results being detected as false positive, or in other words, 96% showed positive results and 4% showed false-positive results.

According to Oliveira et al. (2018), molecular analysis for the detection of Salmonella using real-time PCR showed considerable sensitivity and reproducibility in the amplification of certain fragments and could measure the presence of Salmonella DNA. Molecular research in species detection has been developed in various forms of modification. A study of the identification of Salmonella using real-time PCR, conducted by Piknowa et al. (2002) on 75 types of Salmonella strains using the SYBR green method gave 100% detectable results. The real-time PCR analysis was performed using the 2 step cycling method: Denaturation 95°C for 45 sec and annealing/extension 60°C for 45 sec.

The genes used in this study were invA genes with primary sequences forward (5’-ATC AGT ACC AGT ATT ATC GTA TTG AT-3’), reverse (5’-TCT GTT TAC CGG GCA TAC CAT-3’). The invA gene as primer was selected because the invA gene is a specific gene to identify Salmonella typhimurium ATCC 14028. The use of invA genes for Salmonella detection using real-time PCR has been developed and validated by Malorny et al. (2003). According to Patel et al. (2006), validation is very important to do in making standard methods to provide accurate results.

The Tm value in the qPCR analysis is influenced by the composition and size of the nucleotides. Fluorescent signals give information when DNA bands begin to separate after the annealing process. Melt curve produces a specific single peak of each band detected. However, in each melt curve analysis, the presence of double peaks can sometimes also occur, but even so, the double peaks that occur do not say that this method is not specific (Dwight 2011).

To evaluate real-time PCR tests to detect pathogens in various matrices, both the sensitivity and specificity of the test need to be determined. Analytical sensitivity of a test has been defined as the smallest amount that can be detected from the analyte in question, while analytical specificity is the extent to which the test does not cross-react with other analytes (Bohaychuk et al. 2006).

Health Supplements are products intended to supplement nutrition. The conclusion that can be drawn from this study is the use of real-time PCR with SYBR Green method to identify Salmonella typhimurium ATCC 14028 in 35 samples giving 100% detected results.

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