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DETECTION OF SALMONELLA TYPHIMURIUM BACTERIA ON BAKERY PRODUCTS SAMPLES USING BOILING ISOLATION TECHNIQUE

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ABSTRACT
Detection of Salmonella typhimurium ATCC 14028 bacteria in bakery product samples by real-time PCR using boiling isolation technique. The basis of this research is to have an impact on economic value in carrying out the confirmation test for Salmonella typhimurium ATCC 14028, where testing is carried out conventionally will require large costs, so it is necessary to innovate in terms of modifying the testing phase so that it is more effective and efficient. The purpose of this study was to see whether the boiling isolation technique could be used for the detection test for Salmonella typhimurium ATCC 14028 on bacterial product samples. The sample in this study consisted of 15 types of bacterial product samples spiked with Salmonella typhimurium ATCC 14028 cultures that had been cultured into phase 2 working cultures. The method used in this study was qPCR analysis using the SYBR Green method. The results of real-time PCR analysis obtained Ct values in the range 7.55 - 8.91 with an average of 8.28 and a Tm value in the range 85.50 - 86.20 with an average of 85.77 Based on these data it can be concluded that the detection of Salmonella typhimurium bacteria ATCC 14028 with real-time PCR using boiling isolation technique can be applied for testing on bakery product samples.

Keywords: Boiling; DNA; Real-Time PCR; Salmonella typhimurium

1. INTRODUCTION

Molecular analysis techniques using PCR were first developed by Mullis in 1986, and since then this technique has become very popular in the world of molecular biology (Mullis et al., 1992). DNA-based molecular analysis techniques are the most reliable DNA species identification techniques in recent times. This method has developed rapidly and is becoming a highly used method in molecular biology. Apart from being a method in species DNA research, this method has also been widely applied in the world of disease diagnosis, forensic applications and detection of pathogenic bacteria in clinical, food and environmental samples. This method works specifically through analyzing specific DNA sequences in the genome of the target organism (Lockley & Bardsley, 2000).

The application of molecular analysis techniques to detect pathogenic bacteria from food and beverage products has been growing rapidly recently. Even though conventional identification techniques are still very much needed to detect pathogenic bacteria from food and beverage products, because this technique is quite tiring and takes relatively more time than molecular techniques, the application of this molecular technique needs to be a balanced material in its

application to produce data. and test results that are faster, sensitive, and more accurate. In general, the principle of DNA extraction is composed of three stages which include cell lysis, DNA extraction and DNA purification, however, the boiling technique is the simplest DNA isolation technique which is widely used in the isolation of microorganisms (Martins et al., 2015; Ribeiro Júnior et al., 2016).

With the development of molecular technology, extraction techniques have also developed, giving birth to various types of DNA isolation techniques, including using enzymatic, chemical, heating or thermal, or mechanical lysis techniques, or a combination of the two (Van Tongeren et al., 2011). Several studies using boiling isolation techniques have been carried out on several bacteria (Ahmed & Dablool, 2017; Dimitrakopoulou et al., 2020), Salmonella (Alves et al., 2016), Clostridium perfringen (Ahsani & Shamsaddini, 2013), yeast (Silva et al., 2012) and human DNA (Swa, 2011) . Therefore, this research can be a reference source in testing the quality of food products from pathogenic bacterial contamination that is harmful to public health. Besides, this method is also expected to be a reference method in similar studies to advance molecular biology testing techniques in detecting species DNA.

2. METHODS

2.1. Materials

The ingredients in this study were meatballs, Buffered Peptone Water (BPW) catalogue CM0505 Oxoid, Tris HCl 0.1%, 50 Molal NaOH, and QuantiNova SYBR Green (Qiagen) PCR Kit.

2.2. Sample Preparation

The samples consisted of 15 samples of bakery products which were spiked with positive cultures of Salmonella typhimurium ATCC 14028 phase 2. Phase 2 bacterial cultures were made by culturing positive cultures of Salmonella typhimurium ATCC 14028 from parent standards to produce working standards. The sample was weighed 25 g and then put in a stomacher bag that has a filter, after which 225 mL of Buffered Peptone Water (BPW) and 1 mL of positive Salmonella typhimurium ATCC 14028 phase 2 spike were added and then homogenized by shaking using a stomacher. The results of the homogenization will then be incubated in an incubator with a temperature of 35-37 °C for 44-48 hours without shaking. After that, remove it from the incubator and proceed to the DNA isolation stage.

2.3. DNA isolation

Samples that have been incubated are then pipetted as much as 1 mL, then put in a 2 mL centrifuge tube and centrifuged for 5 minutes at a speed of 14,000 rpm. Discard the supernatant, then the pellet formed at the bottom of the tube was added with 1 mL of 50 molal NaOH then vortexed for 1-2 minutes to dissolve the pellet formed at the bottom of the tube. The sample was then boiled at 100 °C for 10-15 minutes. After boiling, remove and cool at room temperature for 10-15 minutes. The sample was then centrifuged again for 5 minutes at a speed of 14,000 rpm. Pipette the supernatant and discard, the pellet contained in the bottom of the tube was then dissolved by adding 100 µL of 0.1% tris HCL. This solution will then be used for the real-time PCR analysis process.

2.4. Real-Time PCR Analysis

Cycling and melt curve analysis was carried out using qPCR (QIAGEN 5 Plex) with the 2-step cycling method: Denaturation 95 °C for 45 seconds and Annealing / Extention 60 °C for 45 seconds. The primer used to detect Salmonella typhimurium was using InvA Forward primer (5'-ATC AGT ACC AGT CGT CTT ATC TTG AT-3 '), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3').

2.5. Reaction Setup Mix

The master mix with a total volume of 10 µL master mix 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 (Sophian et al., 2021a, 2021b).

2.6. Negative Control

The negative control is NTC (No Template Control), namely master mix combined with primer and nucleic acid-free water. 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 (Sapiun et al., 2020; Sophian et al., 2021a, 2021b).

2.7. Data Analysis

Data analysis was carried out based on 2 main criteria which include: (1) Ct analysis (Cycle threshold), which is to see the Ct value of the sample (2) analysis of melting temperature (Tm), which is at what temperature the melt occurs (Purwaningsih et al., 2020; Sapiun et al., 2020; Sophian et al., 2021b).

3. RESULTS AND DISCUSSION

3.1. Enrichment Results on Media Enrichment Broth

The enrichment process of the enrichment media after incubation for 48 hours at 35-37 ° C using an incubator with the results as presented in (**Table 1**) below.

Table 1. Enrichment result data on enrichment media

Sample	Enrichment Media	After incubation
1-15	BPW	Cloudy

From (**Table 1**) above it can be seen that all the results of the pre-enrichment on the enrichment medium showed 100% turbid results, which means that all the target bacteria spiked on the 20 meatball samples experienced enrichment or growth. This is in line with BAM (2007) which states that the successful pre-enrichment process is marked by the occurrence of turbidity on the BPW media.

3.2. Real-Time PCR Analysis

Real-time PCR analysis was performed using the qualitative method SYBR green and the results were obtained as presented in (**Table 2**). In the Ct analysis, the average Ct value was 8.28, while the Tm analysis obtained an average Tm value of 85.77.

Table 2. Real-Time PCR Data Analysis

Sample	Tm value	Ct value
1	86.00	7.92
2	85.70	7.62
3	86.20	7.95
4	86.00	7.55
5	86.00	8.48
6	85.50	7.94
7	86.00	8.91
8	86.00	7.55
9	85.50	7.55
10	85.50	7.95
11	85.50	7.95
12	86.00	8.91
13	86.00	8.91
14	86.00	8.48
15	85.50	8.48
Average	**85.77**	**8.28**

Molecular analysis using real-time PCR was initiated by isolating the template DNA using the boiling isolation technique. This technique uses the principle of boiling so that the bacterial cell wall breaks and releases DNA which will then be separated using a centrifuge. The advantage of this technique over the centrifuge column or magnetic beads extraction technique is that the cost used is much cheaper and the test time required is less. Meanwhile, when compared to the direct PCR technique, this technique has advantages in the DNA template used, where if using the direct inhibitor technique, it will affect the master mix when reading with real-time PCR while in the boiling technique, DNA has undergone separation with inhibiting factors. in real-time PCR analysis.

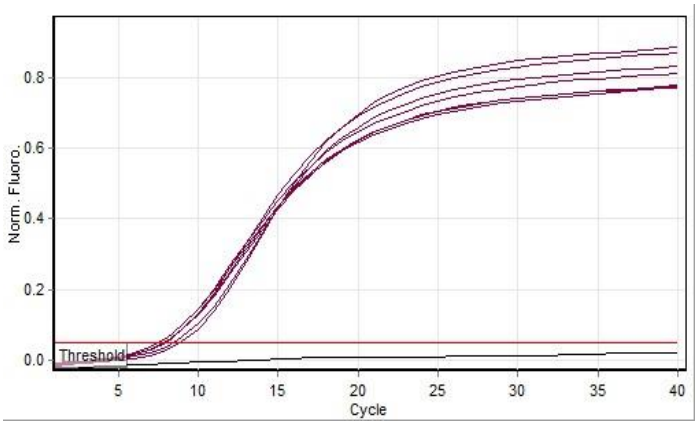

Figure 1. The results of CT analysis of *Salmonella typhimurium* using the boiling isolation method

The results of cycling analysis by looking at the Ct value, show that in the qPCR analysis using the direct method, Salmonella Typhimurium ATCC 14028 was detected at Ct 7.55 - 8.91 as shown in (**Figure 1**). In contrast to analyzing the melt curve, in cycling analysis, the concentration of template DNA used greatly influenced the detected Ct value, while for melt it did not depend on the concentration of template DNA. The amount of concentration can be indicated by the lower value of Ct, or it can also be seen from the smaller the value of Ct, which means the higher the template DNA. Ct analysis cannot be used to see species variation because the Ct value is a cycle value that indicates an amplification.

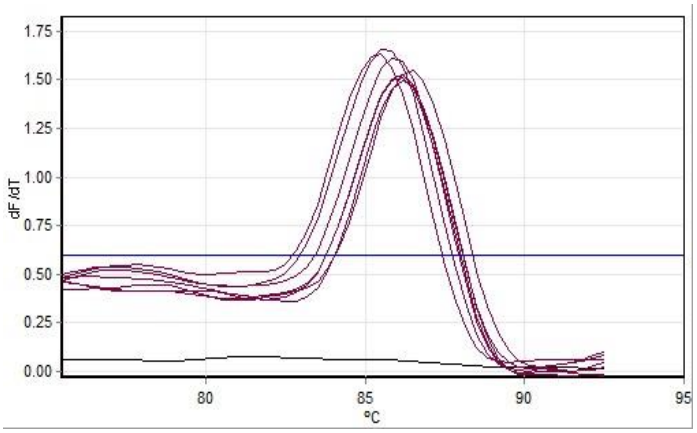

Figure 2. The results of Tm analysis for Salmonella Typhimurium used the boiling isolation method

The results of melt curve analysis by looking at the Tm value show that in the real-time PCR analysis using the direct Salmonella typhimurium ATCC 14028 method was detected at Tm 85.50 - 86.20 as shown in (**Figure 2**). Analysis of Tm or melt curve is associated in a temperature range of 50 °C to 92 °C. In the melt analysis process, when the temperature is low, the DNA will form a double camel by absorbing SYBR green dye. When heating occurs with increasing heating temperature, the double strands will denature. The temperature at which 50% of the double strands

are hybridized is known as the melting temperature (Tm). This temperature has specific characteristics between each species or between each type of primer used because this temperature point is influenced by the length of the primary sequence and the GC content of its nucleotide constituents.

The bacterial DNA extraction technique using the boiling method does not experience significant problems, but this technique will have difficulties when the concentration of targeted bacteria in the sample is very small or because of inhibiting factors for the analysis process using real-time PCR, namely food matrices such as fat, protein, polysaccharides, and calcium. Or it can also be concluded that the characteristics of the samples used also influence the selection of DNA extraction techniques, for example physical, chemical, or biological characteristics of the sample to be used (Giacomazzi et al., 2005; Wilson, 1997). Therefore, in this test stage, a pre-enrichment was carried out on the Buffered Peptone Water (BPW) media of the CM0505 Oxoid catalogue. This stage aims to revive bacteria that are in a dormant state in a sample or to increase bacteria with small concentrations. In the DNA isolation technique, which is carried out by boiling, cell lysis occurs during heating where the nucleic acid from the target will accumulate at the bottom of the tube during centrifugation. This technique has an advantage when compared to the direct PCR technique in which the direct technique does not carry out the extraction or DNA isolation steps so that the amplification process is not as good as if it is done using the boiling technique.

The sample used in this study was the bakery product. The selection of this type of sample is because one of the parameters of FDA supervision in this food category is the identification of Salmonella sp. So, it is hoped that in the future this analysis technique can be used to monitor food products in circulation using molecular analysis techniques using real-time PCR with boiling isolation techniques. In bakery products, the process of making unhygienic products can be a source of contamination with pathogenic bacteria. Bacteria are a type of foodborne disease bacteria that can grow and reproduce at a temperature of 15-14 °C and the optimum growth temperature is 37 °C. If the processing of bakery products is carried out properly, the contamination of pathogenic bacteria will not occur because this product is made using high heating so that the pathogenic bacteria can die during the roasting process.

The primer Salmonella spp. target gene used was the invA gene with the Forward sequence (5'-ATC AGT ACC AGT CGT CTT ATC TTG AT-3 '), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3'). The selection of the invA gene as a specific gene for Salmonella detection was based on a study conducted by (Guerra et al., 2003), where they validated the specificity of the invA target gene for detection of Salmonella. In real-time PCR analysis, primary design techniques play an important role in determining the success of real-time PCR analysis and in increasing the accuracy of the detection process. There are several factors that can be considered in making the primary design, including Length = 18 - 24, Tm = 58 -60, GC content% = 48 -60, Self-complementary = 0.00 - 4.00, Self 3 'complementary = 0.00 - 4.00 and initial base. and Final Bases are not pairable Bases (A / T - G / C). The real-time PCR kit used is the Quantinova SYBR Green kit. The choice of this type of kit is because this kit is much cheaper if we use a kit with a probe system. This SYBR Gree kit works with the principle of 2 step cycling methods: Denaturation 95 ºC for 45 seconds and Annealing / Extention 60 ºC for 45 seconds (Sophian et al., 2021a).

In the DNA amplification process using real-time PCR, at least several materials are mandatory for the successful amplification process, including DNA polymerase, dNTP, Buffer and MgCl2 enzymes as well as forward primers and reverse primers. Primers have a role in the amplification initiation process when entering the annealing phase. If in the annealing stage, the primer does not attach to the target DNA, the amplification process will not take place. To increase the success of amplification, a specific primer is needed. DNA polymerase is an enzyme that has a role in catalyzing reactions to DNA formation. DNTP is Deoxynucleoside triphosphates, which is a mixture consisting of dATP (deoxyadenosine triphosphate), dTTP (deoxythymidine

triphosphate), dCTP (deoxycytidine triphosphate) and dGTP (deoxyguanosine triphosphate) which function as DNA building blocks needed in the process of DNA extension. Buffer is a solution that functions to maintain the pH of the medium, helps stabilize the DNA polymerase enzyme, affects the work of the enzyme, and/or DNA melting temperature (Tm). MgCl2 is a metal ion that functions as an enzymatic cofactor, without MgCl2 DNA polymerase cannot work in the PCR amplification process.

4. CONCLUSION

Based on the results of the research conducted, it was found that Salmonella typhimurium was detected, so the method used in this study can be used as a source of information in similar studies. Suggestions for further research is that it is necessary to validate the method used so that the reliability of this method can be tested to be used as a standard method in testing the detection of Salmonella typhimurium in samples of bakery products.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

7. REFERENCES

Ahmed, O. B., & Dablool, A. S. (2017). Quality improvement of the DNA extracted by boiling method in gram negative bacteria. *International Journal of Bioassays*, *6*(4), 5347–5349.

Ahsani, M. R., & Shamsaddini, B. M. (2013). *Compare of two methods of direct PCR and PCR with DNA extraction in Clostridium perfringens typing*.

Alves, J., Hirooka, E. Y., & de Oliveira, T. C. R. M. (2016). Development of a multiplex real-time PCR assay with an internal amplification control for the detection of Campylobacter spp. and Salmonella spp. in chicken meat. *LWT-Food Science and Technology*, *72*, 175–181.

Dimitrakopoulou, M.-E., Stavrou, V., Kotsalou, C., & Vantarakis, A. (2020). Boiling extraction method VS commercial Kits for bacterial DNA isolation from food samples. *Journal of Food Science and Nutrition Research*, *3*(4), 311–319.

Giacomazzi, S., Leroi, F., & Joffraud, J. (2005). Comparison of three methods of DNA extraction from cold-smoked salmon and impact of physical treatments. *Journal of Applied Microbiology*, *98*(5), 1230–1238.

Guerra, B., Junker, E., Schroeter, A., Malorny, B., Lehmann, S., & Helmuth, R. (2003). Phenotypic and genotypic characterization of antimicrobial resistance in German Escherichia coli isolates from cattle, swine and poultry. *Journal of Antimicrobial Chemotherapy*, *52*(3), 489–492.

Lockley, A. K., & Bardsley, R. G. (2000). DNA-based methods for food authentication. *Trends in Food Science & Technology*, *11*(2), 67–77.

Martins, F. H., Guth, B. E. C., Piazza, R. M., Leao, S. C., Ludovico, A., Ludovico, M. S., Dahbi, G., Marzoa, J., Mora, A., & Blanco, J. (2015). Diversity of Shiga toxin-producing Escherichia coli in sheep flocks of Paraná State, southern Brazil. *Veterinary Microbiology*, *175*(1), 150–156.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1992). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Biotechnology Series*, 17.

Purwaningsih, R., Lukita, B. L., & Ningsih, E. C. (2020). Detection of Salmonella typhimurium ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR). *Asian Journal of Natural Product Biochemistry*, *18*(2).

Ribeiro Júnior, J. C., Tamanini, R., Soares, B. F., de Oliveira, A. M., Silva, F. G., da Silva, F. F., Augusto, N. A., & Beloti, V. (2016). *Efficiency of boiling and four other methods for genomic DNA extraction of deteriorating spore-forming bacteria from milk. Semin. Cienc.*

Agrar. *37: 3069–3078*.

Sapiun, Z., Sophian, A., Abinawanto, M., Kamba, V., Damiti, S. A., & Luawo, H. (2020). Optimization of McFarland Turbidity Standards Value in Determining Template DNA as Reference in Salmonella typhimurium ATCC 14028 Test Using Real-Time PCR (qPCR). *PalArch's Journal of Archaeology of Egypt/Egyptology*, *17*(6), 10916–10922.

Silva, G. A. da, Bernardi, T. L., Schaker, P. D. C., Menegotto, M., & Valente, P. (2012). Rapid yeast DNA extraction by boiling and freeze-thawing without using chemical reagents and DNA purification. *Brazilian Archives of Biology and Technology*, *55*, 319–327.

Sophian, A., Purwaningsih, R., Muindar, M., Igirisa, E. P. J., & Amirullah, M. L. (2021a). Detection of Salmonella typhimurium ATCC 14028 in Powder Prepared Traditional Medicines Using Real-Time PCR. *Borneo Journal of Pharmacy*, *4*(3), 178–183.

Sophian, A., Purwaningsih, R., Muindar, M., Igirisa, E. P. J., & Amirullah, M. L. (2021b). Use of Direct PCR Technique Without DNA Extraction in Confirmation Test for Salmonella typhimurium Bacteria on Meatball Samples. *Borneo Journal of Pharmacy*, *4*(4), 324–332.

Swa, Y. C. (2011). Successful Generation of DNA profiles using Direct PCR. *Infrared Spectroscopic Technique for the Forensic Discrimination of Marker*.

Van Tongeren, S. P., Degener, J. E., & Harmsen, H. J. M. (2011). Comparison of three rapid and easy bacterial DNA extraction methods for use with quantitative real-time PCR. *European Journal of Clinical Microbiology & Infectious Diseases*, *30*(9), 1053–1061.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, *63*(10), 3741–3751.