Research Article

Use of Direct PCR Technique Without DNA Extraction in Confirmation Test for *Salmonella typhimurium* Bacteria on Meatball Samples

Alfi Sophian [1*]

Ratna Purwaningsih [1]

Muindar [2]

Eka Putri Juniarti Igirisa [2]

Muhammad Luthfi Amirullah [2]

[1]National Agency of Drug and Food Control of Republic of Indonesia, Central Jakarta, Special Capital Region of Jakarta, Indonesia

[2]National Agency of Drug and Food Control of Republic of Indonesia in Gorontalo, Bone Bolango, Gorontalo, Indonesia

*email: alfi.sophian@pom.go.id

Abstract

The use of direct PCR technique without DNA extraction in the confirmation test for *Salmonella typhimurium* ATCC 14028 bacteria on meatball samples was carried out in the Food and Drug molecular biology testing laboratory Administration in Gorontalo. The basis of this research is to have an impact on economic value in carrying out the confirmation test for *S. typhimurium* ATCC 14028, where testing is carried out conventionally, namely DNA extraction, which requires a large amount of money. Hence, it is necessary to innovate to modify the testing phase so that it is more effective and efficient. The purpose of this study was to see whether the direct PCR technique without DNA extraction can be done for the confirmation test of *S. typhimurium* ATCC 14028 on meatball samples. This study's sample consisted of 20 types of meatball samples spiked with *S. typhimurium* ATCC 14028 cultures. The method used in this study was qPCR analysis using the SYBR Green method. Data analysis was carried out based on two main criteria: (1) Ct analysis and (2) Tm analysis. Real-time PCR analysis results obtained Ct values in the range 14.14 - 15.20 with an average of 14.82 and Tm values 85.20 – 86.30 with an average of 85.79. Based on these data, it can be concluded that using direct PCR can be used for testing confirmation of *S. typhimurium* ATCC 14028 on meatball samples.

INTRODUCTION

Direct polymerase chain reaction (PCR) is a PCR technique without deoxyribonucleic acid (DNA) extraction which is carried out to detect pathogenic bacteria quickly and accurately[1]. The direct PCR technique has been carried out by several researchers in detecting *Escherichia coli*, Lactic acid bacteria, and nonpathogenic *Staphylococci* from artisanal low-acid sausages[2,3]. Research using the direct technique is very cost-effective research, where the cost of purchasing an extraction kit which is quite expensive, can be reduced by this technique. In addition, this technique can save testing time where the long and time-consuming incubation process can be reduced[4]. Research conducted by Aymerich *et al.*[3] on *E. coli* without pre-enrichment media showed that this method could be used as a confirmation test method and as a direct detection test method to save time and cost of the testing process. Several researchers have also investigated by not doing the enrichment stage[5-8].

The confirmation test for *Salmonella typhimurium* was carried out to replace the conventional confirmation test technique that has been carried out in the detection of *S. typhimurium*, which in conventional technique uses a

biochemical system[9]. In contrast, this is a molecular-based confirmation test technique using real-time quantitative PCR (qPCR)[10]. The direct PCR method was chosen to reduce the testing time and the cost of testing, which is increasingly expensive compared to conventional techniques by using the extraction step before performing PCR analysis[11].

According to Holt *et al.*[12], *S. typhimurium* is a Gram-negative bacterium belonging to the Enterobacteriaceae family, rod-shaped, non-spore-forming, and usually motile. *Salmonella typhimurium* is a bacterial pathogen usually found in animals and humans. The cell wall is composed of lipopolysaccharide, which can cause endotoxin effects in infected animals and humans[13]. Several techniques are commonly used to detect *S. typhimurium* in food products, including conventional techniques using selective agar media, biochemical confirmation tests, and molecular techniques, which are becoming more popular because they are relatively more practical and shorter in processing[14,15]. According to Postollec *et al.*[16], species identification using qPCR is a fast and reliable alternative method in the development of the molecular world. At least two qPCR methods are often used in conducting analyses to detect species DNA, namely the TaqMan® and SYBR® Green methods[17-21].

Meatballs are food products made from processed animal meat. This study selected meatball samples because *S. typhimurium* is one of the bacteria found in animal meat products whose contamination can come from the environment or an unhygienic slaughter process[22]. Meatballs are meat-processed food products that are much favored by the public. The high level of bacterial contamination in meatballs has been revealed by Zain *et al.*[23], wherein their research found that *S. typhimurium* detected 20% of the samples of meatballs used in their research. Our previous study[24] conducted several studies on detecting *S. typhimurium* in meatball

products using a rapid test kit. However, using qPCR techniques in *S. typhimurium* detection testing is a step forward in identifying/detecting pathogenic bacteria to produce a faster test time than conventional techniques or more accurate when compared to rapid test kits.

The biggest challenge in controlling food products in circulation is how to produce test results data quickly with the lowest possible testing costs. Therefore, this research was conducted to serve as a reference in confirming the detection of *S. typhimurium* in meatball products so that it can be used in food quality testing to identify contamination of pathogenic bacteria that can be used in food quality testing to identify contamination of pathogenic bacteria contaminates food products.

MATERIALS AND METHODS

Materials

The sample in this study were meatballs without particular criteria and generally sold by traders who were sampled from several different locations and then processed in the laboratory by a spike with positive control *S. typhimurium* ATCC 14028. Other materials including buffered peptone water (BPW) (CM0509 Oxoid), Xylose Lysine Deoxycholate (XLD) agar (CM0469 Oxoid), Hektoen Enteric Agar (HEA) (CM0419 Oxoid), Tryptone Soya Agar (TSA) (CM0131 Oxoid), sterile aquadest, McFarland standard No.2, QuantiNova SYBR® Green PCR Kit (Qiagen), micropipette (Eppendorf), and qPCR Rotor-Gene Q 5plex (Qiagen).

Methods

Media setup

The BPW liquid media was made by weighing 20 g of BPW media, adding 1 L of distilled water, dissolved using a stirrer, and then sterilized by autoclave at 121°C for 15 minutes. The HEA agar medium was made by weighing 76 g of HEA media, then dissolved using distilled water that had been sterilized first and then

heated, which would then be used to dissolve the HEA media. Media for HEA should not be autoclaved. Likewise, liquid media and other agar were made according to the instructions for existing media.

Sample preparation

The sample consisted of 20 meatball samples spiked with positive cultures of *S. typhimurium* phase 2. Phase 2 bacterial cultures were made by sifting positive cultures of *S. typhimurium* from master standards to produce working standards. The sample was weighed 25 g, added 225 mL of BPW and 1 mL of positive contact spike *S. typhimurium* phase 2, then homogenized by shaking using a stomacher. The homogenization results were then incubated in an incubator with a temperature of 35-37°C for 24 hours. After 24 hours, the samples were scratched on selective media HEA and XLD agar, then incubated at 35-37°C for 24 hours. The colony interpretation results on selective media were recorded, then select specific colonies were to be scratched on slant TSA media and re-incubated in an incubator with a temperature of 35-37°C for 24 hours. After 24 hours, the incubation results on the TSA were then taken a loop and turbid in sterile distilled water in which the turbidity was equalized to McFarland standard No.2. The results of equalization were then used as DNA templates.

DNA isolation

DNA isolation for the direct technique was not carried out because the enrichment from the working culture was directly scratched on the medium to make it slant. The growing colonies were then dissolved in physiological NaCl, and the turbidity level was equalized to McFarland standard No.2. The result of equalization was then used as the template DNA.

qPCR analysis

Cycling and melt curve analysis was carried out using qPCR Rotor-Gene Q 5plex 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')[25,26].

Reaction setup

The master mix was mixed with a total volume of 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[27,28].

Negative control

The negative control was NTC (no template control), a master mix combined with primer and nucleic acid-free water. The total negative control volume was 10 µL, consisting of 5 µL master mix SYBR® Green, 1 µL forward primer, 1 µL reverse primer, and 3 µL RNase-free water[27].

Data analysis

Data analysis was carried out based on two main criteria: (1) cycle threshold (Ct) analysis and (2) analysis of melting temperature (Tm), as described in our previous study[27].

RESULTS AND DISCUSSION

Enrichment on media enrichment broth

The enrichment process of the enrichment media after incubation for 24 hours at 35-37°C using an incubator with the results as presented in **Table I**. 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 result was in line with the USFDA Bacteriological Analytical Manual (BAM)[29], which states that the success of the pre-enrichment process is marked by the occurrence of turbidity in the BPW media.

Table I. Enrichment result data on enrichment media

Meatball sample number	Enrichment media	After incubation
1	BPW	Cloudy
2	BPW	Cloudy
3	BPW	Cloudy
4	BPW	Cloudy
5	BPW	Cloudy
6	BPW	Cloudy
7	BPW	Cloudy
8	BPW	Cloudy
9	BPW	Cloudy
10	BPW	Cloudy
11	BPW	Cloudy
12	BPW	Cloudy
13	BPW	Cloudy
14	BPW	Cloudy
15	BPW	Cloudy
16	BPW	Cloudy
17	BPW	Cloudy
18	BPW	Cloudy
19	BPW	Cloudy
20	BPW	Cloudy

Table II. Isolation result on selective media after incubation

Meatball sample number	XLD	HEA
1	translucent spherical with black spots	greenish-blue
2	translucent spherical with black spots	greenish-blue
3	translucent spherical with black spots	greenish-blue
4	translucent spherical with black spots	greenish-blue
5	translucent spherical with black spots	greenish-blue
6	translucent spherical with black spots	greenish-blue
7	translucent spherical with black spots	greenish-blue
8	translucent spherical with black spots	greenish-blue
9	translucent spherical with black spots	greenish-blue
10	translucent spherical with black spots	greenish-blue
11	translucent spherical with black spots	greenish-blue
12	translucent spherical with black spots	greenish-blue
13	translucent spherical with black spots	greenish-blue
14	translucent spherical with black spots	greenish-blue
15	translucent spherical with black spots	greenish-blue
16	translucent spherical with black spots	greenish-blue
17	translucent spherical with black spots	greenish-blue
18	translucent spherical with black spots	greenish-blue
19	translucent spherical with black spots	greenish-blue
20	translucent spherical with black spots	greenish-blue

Isolation on selective media

The results of isolation on selective media after incubation for 24 hours at 35-37°C using an incubator were presented in **Table II**. All samples identified specific characteristics of *S. typhimurium* on selective media, in which the XLD target bacteria had translucent spherical colony characteristics with black spots in the middle. In contrast, the colonies were greenish-blue with or without the black color at the colony's center in HEA. These characteristics of the colony growth indicate that all isolated samples could grow *S. typhimurium* as target bacteria.

The initial stage of this research was the pre-enrichment of all samples on BPW. All samples were then scratched on selective media XLD agar and HEA. Pre-enrichment is a step carried out to enrich or increase the volume or concentration of target bacteria. This stage is essential because the enrichment that occurs makes bacteria dormant in the sample matrix return to life and carry out their biological activities so that when scratched on selective media, the target bacteria to be identified can easily grow[30]. The success of the pre-enrichment stage is characterized by turbidity and a characteristic odor in the incubated samples[29].

Real-time PCR analysis

Real-time PCR analysis was performed using the qualitative method SYBR® Green, and the results were obtained as presented in **Figure 1**. The mean Ct value was 14.82 cycles in the Ct analysis, while the Tm analysis obtained an average Tm value of 85.79°C. Real-time PCR analysis performed using the direct technique resulted in no DNA extraction stage. This made the detected sample not have the consistent concentration of template DNA used so that the pattern of the cycling or melt curve was slightly irregular. In this direct technique, the template DNA was mixed with the inhibitor originating from the bacterial cell wall, which did not undergo settlement due

to the absence of a DNA extraction stage. The advantage of this technique is that the time required to produce data is much faster[31]. A study reported by Nugraha *et al.*[32] in *Salmonella* sp. detection using real-time PCR on fishery product samples obtained a Tm value of 86.60°C. Several studies have found that in the detection of *Salmonella* sp., Tm values were usually only shifted 1-2°C. This finding shows that the Tm results obtained were similar to previous research[32,33].
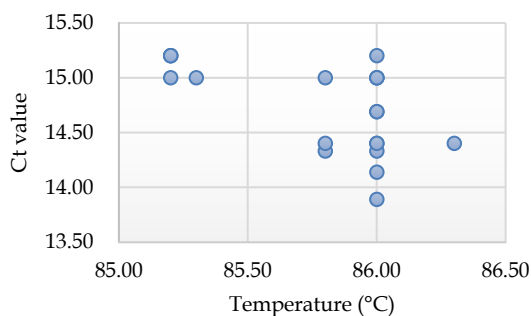

Figure 1. Real-time PCR data analysis

The Ct value's cycling analysis shows that in the direct method's qPCR analysis, *S. typhimurium* was detected at Ct 4.14 - 15.20, as shown in **Figure 2**. The value of Ct in real-time PCR analysis was influenced by several factors, including the number of nucleotides that comprise it and the concentration of the template DNA used. The higher the concentration of the template DNA used, the smaller the Ct value, and vice versa. The exponential phase is essential in seeing the success of the amplification process, wherein this phase and increase in PCR products will be seen significantly. The higher the template concentration used, the faster the fluorescent emission detection would be at the initial Ct[26,34,35]. Sapiun *et al.*[28] reported differently, in their research that optimized variations in the concentration of DNA template used for the confirmation test of *Salmonella* spp., the results were not significantly different.
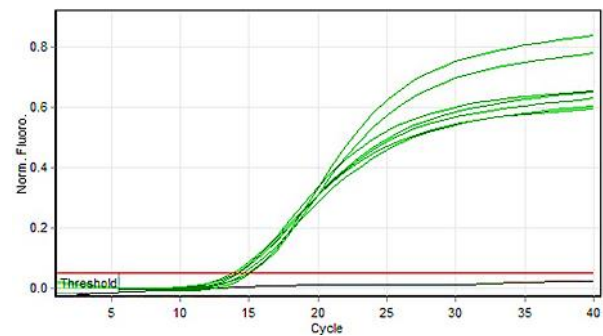

Figure 2. The Ct analysis of *S. typhimurium* using the direct PCR method (green: sample; black: negative control)

The results of melt curve analysis by looking at the Tm value show that in the qPCR analysis using the direct *S. typhimurium* method was detected at Tm 85.20 - 86.30°C, as shown in **Figure 3**. In principle, Tm analysis works by looking at the temperature at which melting occurs. As with cycling analysis, the curve pattern in melt curve analysis is also influenced by several factors, including the length of the DNA primer sequences used and the guanine-cytosine content (GC content) that compose the primers used[36].
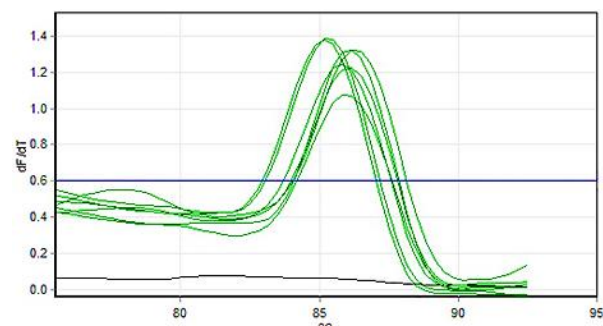

Figure 3. The Tm analysis of *S. typhimurium* using the direct PCR method (green: sample; black: negative control)

The best molecular analysis technique used to detect pathogenic *S. typhimurium* by far is molecular-based analysis with real-time PCR[33]. Direct PCR has a very economic advantage compared to molecular techniques requiring DNA extraction in the analysis process[37]. One of the PCR analysis methods, which is also economical, is the SYBR® Green method. This method is performed with a two-step cycling method: denaturation 95°C for 45 seconds and annealing/extension 60°C for 45 seconds[38].

The SYBR® Green technique is an amplification technique that uses the Tm value as a reference for a test wherein the difference in the value range of 2 degrees is still considered a fair value[25]. This distinction was made because although the influencing factor: GC content in the Tm analysis, inhibitor factors, and concentration variations must also be considered factors that can affect the accuracy of a test process[39]. Combining the direct PCR technique with the SYBR® Green method in the molecular analysis is economical[38,40].

The primers used in this study were the invA gene with the forward primary 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 the primary was used because the invA gene was a specific gene to identify *S. typhimurium*. The invA gene was used to detect *S. typhimurium* using real-time PCR, developed and validated by Malorny *et al*[41].

In real-time PCR analysis using the melt curve SYBR® Green method, the Tm value can be influenced by the composition and size of the nucleotides that make up DNA. The use of the SYBR® Green master mix also affects the workings of the real-time PCR analysis system, where the fluorescence signal in the SYBR® Green method will provide information when the DNA bands begin to separate after the annealing process. The melt curve works by producing a specific single peak from each band detected with amplified species of nucleotide components. Not all analysis processes are perfect, as well as melt curve analysis. The error that generally occurs in this method is the presence of double peaks that appear after the amplification analysis process. However, the double peaks that occur do not say that this method is not specific[42].

According to Bohaychuk *et al*.[43], to evaluate molecular-based testing using real-time PCR in detecting pathogenic bacteria, it is necessary to pay attention to the matrix composing the sample to be tested to see whether the matrix has an effect or not. Besides that, it is also necessary to test the specificity and sensitivity. If all research stages have been carried out, the reliability of the test method produced can be measured in conducting the test.

This research is exciting because it uses methods that have been used in several commodities, including health supplement products and traditional medicines[24,26]. In this study, the challenge that is slightly different from previous research is that the use of different types of samples undoubtedly affects the ability of the tests carried out. The success of this study in detecting meatball samples will make the method being developed known for its reliability so that it can continue to grow and develop—used for other types of samples without significant modification.

CONCLUSION

The use of direct PCR technique without DNA extraction in the confirmation test of *S. Typhimurium* on meatball samples was successfully carried out.

ACKNOWLEDGMENT

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.

REFERENCES

1. Louie M, Louie L, Simor AE. The role of DNA amplification technology in the diagnosis of infectious diseases. CMAJ. 2000;163(3):301-9. doi:10.1016/s1381-1169(00)00220-x

2. Kotsiri Z, Vantarakis A, Rizzotto F, Kavanaugh D, Ramarao N, Vidic J. Sensitive Detection of E. coli in Artificial Seawater by Aptamer-Coated Magnetic Beads and Direct PCR. Appl Sci. 2019;9(24):5392. doi:10.3390/app9245392

3. Aymerich T, Martín B, Garriga M, Hugas M. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic Staphylococci from artisanal low-acid sausages. Appl Environ Microbiol. 2003;69(8):4583-94. doi:10.1128/aem.69.8.4583-4594.2003

4. Morecchiato F, Coppi M, Baccani I, Maggini N, Ciccone N, Antonelli A, et al. Evaluation of extraction-free RT-PCR methods for faster and cheaper detection of SARS-CoV-2 using two commercial systems. Int J Infect Dis. 2021;112:264-8. doi:10.1016/j.ijid.2021.09.046

5. Xiao L, Zhang Z, Sun X, Pan Y, Zhao Y. Development of a quantitative real-time PCR assay for viable Salmonella spp. without enrichment. Food Control. 2015;57:185-9. doi:10.1016/j.foodcont.2015.03.050

6. Miller ND, Davidson PM, D'Souza DH. Real-time reverse-transcriptase PCR for Salmonella Typhimurium detection from lettuce and tomatoes. LWT - Food Sci Technol. 2011;44(4):1088-97. doi:10.1016/j.lwt.2010.08.003

7. D'Urso OF, Poltronieri P, Marsigliante S, Storelli C, Hernández M, Rodríguez-Lázaro D. A filtration-based real-time PCR method for the quantitative detection of viable Salmonella enterica and Listeria monocytogenes in food samples. Food Microbiol. 2009;26(3):311-6. doi:10.1016/j.fm.2008.12.006

8. Elizaquível P, Aznar R. A multiplex RTi-PCR reaction for simultaneous detection of Escherichia coli O157:H7, Salmonella spp. and Staphylococcus aureus on fresh, minimally processed vegetables. Food Microbiol. 2008;25(5):703-13. doi:10.1016/j.fm.2008.03.002

9. Andrews JR, Ryan ET. Diagnostics for invasive Salmonella infections: current challenges and future directions. Vaccine. 2015;33(Suppl 3):C8-C15. doi:10.1016/j.vaccine.2015.02.030

10. Ranjbar R, Mortazavi SM, Tavana AM, Sarshar M, Najafi A, Zanjani RS. Simultaneous Molecular Detection of Salmonella enterica Serovars Typhi, Enteritidis, Infantis, and Typhimurium. Iran J Public Health. 2017;46(1):103-11.

11. Yang S, Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 2004;4(6):337-48. doi:10.1016/s1473-3099(04)01044-8

12. Holt JG, Krieg NR, Sneath PHA, Stanley JT, William ST. Bergey's Manual of Determinative Bacteriology. 9th edition. Baltimore (MD): Lippincott Williams & Wilkins; 2000.

13. Ramachandran G. Gram-positive and gram-negative bacterial toxins in sepsis. Virulence. 2014;5(1):213-8. doi:10.4161/viru.27024

14. Bell RL, Jarvis KG, Ottesen AR, McFarland MA, Brown EW. Recent and emerging innovations in Salmonella detection: a food and environmental perspective. Microb Biotechnol. 2016;9(3):279-92. doi:10.1111/1751-7915.12359

15. Adzitey F, Huda N, Ali GRR. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. 3 Biotech. 2013;3(2):97-107. doi:10.1007/s13205-012-0074-4

16. Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol. 2011;28(5):848-61. doi:10.1016/j.fm.2011.02.008

17. Beutin L, Jahn S, Fach P. Evaluation of the 'GeneDisc' real-time PCR system for detection of enterohaemorrhagic Escherichia coli (EHEC) O26, O103, O111, O145 and O157 strains according to their virulence markers and their O- and H-antigen-associated genes. J Appl Microbiol. 2009;106(4):1122-32. doi:10.1111/j.1365-2672.2008.04076.x

18. Botteldoorn N, Heyndrickx M, Rijpens N, Herman L. Detection and characterization of verotoxigenic Escherichia coli by a VTEC/EHEC multiplex PCR in porcine faeces and pig carcass swabs. Res Microbiol. 2003;154(2):97-104. doi:10.1016/s0923-2508(03)00028-7

19. Fukushima H, Katsube K, Tsunomori Y, Kishi R, Atsuta J, Akiba Y. Comprehensive and rapid real-time PCR analysis of 21 foodborne outbreaks. Int J Microbiol. 2009;2009:917623. doi:10.1155/2009/917623

20. Kim HJ, Cho JC. Simple and rapid detection of Listeria monocytogenes in fruit juice by real-time PCR without enrichment culture. Food Control. 2010;21(10):1419-23. doi:10.1016/j.foodcont.2010.04.006

21. Singh J, Batish VK, Grover S. Simultaneous detection of Listeria monocytogenes and Salmonella spp. in dairy products using real time PCR-melt curve analysis. J Food Sci technol. 2012;49(2):234-9. doi:10.1007/s13197-011-0278-3

22. Rose BE, Hill WE, Umholtz R, Ransom GM, James WO. Testing for Salmonella in raw meat and poultry products collected at federally inspected establishments in the United States, 1998 through 2000. J Food Prot. 2002;65(6):937-47. doi:10.4315/0362-028x-65.6.937

23. Zain R, Hidanah S, Damayanti R, Warsito SH. Detection of Salmonella sp. on bulk meatballs and packaged meatballs at Sepanjang Market, Sidoarjo. J Appl Vet Sci Technol. 2021;2(2):31-6. doi:10.20473/javest.V2.I2.2021.31-36

24. Sophian A, Muindar. Pengujian Salmonella typhimurium ATCC 14028 pada produk sosis, nugget, bakso, otak-otak, tempura dan cilok menggunakan kit rapid test. J Exp Clin Pharm. 2021;1(1):48-55. doi:10.52365/jecp.v1i1.198

25. Sophian A, Purwaningsih R, Igirisa EPJ, Amirullah ML, Lukita BL, Fitri RA. Short Communication: Detection of Salmonella typhimurium ATCC 14028 and Listeria monocytogenes ATCC 7644 in processed meat products using Real-Time PCR Multiplex Method. Asian J Nat Prod Biochem. 2021;19(1):17-20. doi:10.13057/biofar/f190103

26. Sophian A, Purwaningsih R, Muindar, Igirisa EPJ, Amirullah ML. Detection of Salmonella typhimurium ATCC 14028 in Powder Prepared Traditional Medicines Using Real-Time PCR. Borneo

J Pharm. 2021;4(3):178-83. doi:10.33084/bjop.v4i3.1838

27. Sophian A, Purwaningsih R, Lukita BL, Ningsih EC. Detection of Salmonella typhimurium ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR). Asian J Nat Prod Biochem. 2020;18(2):65-6. doi:10.13057/biofar/f180202

28. Sapiun Z, Kamba V, Damiti SA, Sophian A, Abinawanto, Muindar, et al. 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 J Archaeol Egypt/Egyptol. 2020;17(6):10916-21.

29. Andrews WH, Wang H, Jacobson A, Ge B, Zhang G, Hammack T. Bacteriological Analytical Manual. BAM Chapter 5: Salmonella. Silver Spring (MD): The United States Food and Drug Administration; 2007 [updated 2021 Oct; cited 2021 Nov]. Available from: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-5-salmonella

30. Baylis CL, MacPhee S, Betts RP. Comparison of two commercial preparations of buffered peptone water for the recovery and growth of Salmonella bacteria from foods. J Appl Microbiol. 2000;89(3):501-10. doi:10.1046/j.1365-2672.2000.01145.x

31. Gupta N. DNA Extraction and Polymerase Chain Reaction. J Cytol. 2019;36(2):116-7. doi:10.4103/JOC.JOC_110_18

32. Nugraha R, Nurilmala M, Nurjanah, Pratama P. Detection of Salmonella sp. in fisheries product using real-time PCR. IOP Conf Ser Earth Environ Sci. 2020;404:012012. doi:10.1088/1755-1315/404/1/012012

33. de Oliveira ACdS, Rosa MC, Bochardt JL, Menegon YA, Fernandes MMA, Cardoso GVF, et al. Validating the Efficiency of a Simplex PCR and Quantitative SYBR Green qPCR for the Identification of Salmonella spp. DNA. J Food Microbiol Saf Hyg. 2018;3:1. doi:10.4172/2476-2059.1000130

34. Dorak M. Real-time PCR. London (UK): Taylor & Francis; 2006. doi:10.4324/9780203967317

35. Karlen Y, McNair A, Perseguers S, Mazza C, Mermod N. Statistical significance of quantitative PCR. BMC Bioinformatics. 2007;8:131. doi:10.1186/1471-2105-8-131

36. Abtahi H, Sadeghi MR, Shabani M, Edalatkhah H, Hadavi R, Akhondi MM, et al. Causes of bimodal melting curve:Asymmetric guaninecytosine (GC) distribution causing two peaks in melting curve and affecting their shapes. Afr J Biotechnol. 2011;10(50:10196-203. doi:10.5897/AJB11.1782

37. Carter I, Halliday C, Sloots TP, Pryce TM, Kay ID, Harnett GB, et al. PCR Methodology. In: Schuller M, Sloots T, James G, Halliday C, Carter I, editors. PCR for Clinical Microbiology. Dordrecht: Springer; 2010. p. 11-47. doi:10.1007/978-90-481-9039-3_2

38. Lorenz TC. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. J Vis Exp. 2012;63:3998. doi:10.3791/3998

39. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet. 2013;54(4):391-406. doi:10.1007/s13353-013-0173-x

40. Wong G, Wong I, Chan K, Hsieh Y, Wong S. A Rapid and Low-Cost PCR Thermal Cycler for Low Resource Settings. PLoS One. 2015;10(7):e0131701. doi:10.1371/journal.pone.0131701

41. Malorny B, Hoorfar J, Bunge C, Helmuth R. Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. Appl Environ Microbiol. 2003;69(1):290-6. doi:10.1128/aem.69.1.290-296.2003

42. Gudnason H, Dufva M, Bang DD, Wolff A. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Res. 2007;35(19):e127. doi:10.1093/nar/gkm671

43. Bohaychuk VM, Gensler GE, McFall ME, King RK, Renter DG. A real-time PCR assay for the detection of Salmonella in a wide variety of food and food-animal matricest. J Food Prot. 2007;70(5):1080-7. doi:10.4315/0362-028x-70.5.1080