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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 accurately1. 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 sausages2,3. 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 reduced4. 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 stage5-8. 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 system9. 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 analysis11. 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 humans13. 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 processing14,15. 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 methods17-21. 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 process22. 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 study24 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 template27,28.

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 water27. 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 study27.

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 _ _ 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 grow30. The success of the pre-enrichment stage is characterized by turbidity and a characteristic odor in the incubated samples29. 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 _ _ 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 faster31. 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 research32,33. / 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 Ct26,34,35. 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 used36. / 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 PCR33. Direct PCR has a very economic advantage compared to molecular techniques requiring DNA extraction in the analysis process37. 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 seconds38.

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 value25. 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 process39. Combining the direct PCR technique with the SYBR® Green method in the molecular analysis is economical38,40.

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 al41. 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 specific42. 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 medicines24,26. 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.

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