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Comparison of Real-Time PCR and Conventional PCR by Identifying Genomic DNA of Bovine and Porcine Ahlam Inayatullah1, Annisa Fatmawati 2, Emelda2, Muhammad Abdurrahman Munir2* 1Faculty of Science and Technology, Universiti Sains Islam Malaysia, Bandar Baru Nilai, Nilai, Negeri Sembi- lan, 71800, Malaysia 2Department of Pharmacy, Faculty of Health Science, Universitas Alma Ata, Bantul, Daerah Istimewa Yogya- karta, 55183, Indonesia *Corresponding Author: muhammad@almaata.ac.id 1. INTRODUCTION Nowadays, analysis of food products is impera - tive to identify the quantity and quality of food, preventing food adulteration and promoting food safety.

Meat species analysis becomes a persistent issue that must be handled for several reasons such (a) quantity meat is compared on the product label, (b) substitute high-quality meats partially, even for some cases convert entirely with low-quality then put counterfeit label deliberately before distributed to markets, (c) the concentration of meat inside non – meat products and (d) to follow the regulation of certain country related to Halal food, where Islamic law stringently prohibits the consumption of specific meat products (e.g., porcine products) (Dolch et al. 2020; Zia et al. 2020; Kang et al. 2021). Those reasons should be considered to satisfy and protect consumers.

There are several approaches to detect meat species in foods, such as genomics (Wang et al. 2019; Sultana et al. 2020), spectroscopy (Sankar et al. 2020; Cebi et al. 2019), chromatography (Ekasary et al. 2018; Sha et al. 2018), morphology (Labrooy et al. 2018; Zhang et al. 2019), immunochemical (Tukiran et al. 2016) and proteomics (Chis & Vodnar 2019; Wang et al. 2019). The genomic ARTICLE INFO Abstract Article History: Received date: 16 June 2021 Revised date: 25 August 2021 Accepted date: 27 September 2021 Available online at: November 2021 Bovine and porcine are poultry meat that is consumed worldwide, particularly in Southeast Asia. Both of them are prone

to food co unterfeit owing to several factors such as price, appetite and Halal status. Sensitive and selective analytical methods are required to co ntrol meat products distributed to markets.

Th is paper studied the sensitivity between real- time and conventional PC R. Bovine and porcine were used as the sample to verify the sensitivity of the method. The result of the study found that the assays did not show a specific difference during DNA analysis of bovine and p orcine. In conventional PCR, two pairs of DNA primers targeted cytochrome b (Cyt b) were analyzed, resulting in 120 and 131 amplicons, respectively. While qPCR was applied to analyze porcine and bovine DNA.

The detection limit of qPCR after por cine and bovine analysis was 0.004 and 0.007 μg/μL, respectively. R esults demonstr ated that the qPCR was reliable for verifying porcine and bovine DNA compared to conventional PCR. Furthermore, the study concluded that the developed assay could easily identify porcine and bovine tissue in food products in low resource areas. © Jurnal Terapan This an access under CC license by-nc-sa/4.0/). Keywords: Bovine, Porcine, PCR, DNA. method with the PCR technique has been used by Li et al. (2021) to detect the species content in the product accurately.

Furthermore, living organ- isms have their own DNA' molecules that very unique for each organism (Williams et al. 2020; Zulch et al. 2020). PCR technique is strongly selective and sensitive by multiplying nucleotide vitro (Toohey – Kurth et al. 2020). DNA sequence parts to be multiplied before the multiplication process can be done. The sequence is imperative to provide a primer where the short oligonucleotide sequence initiates the DNA synthesis in a polymerase chain reaction (Li et al. 2020). Furthermore, the reaction is followed by a heating machine that provides thermal conditions amplification (Mancini et al. 2020).

The process inside the PCR machine is divided into three steps such as denaturation (double-stranded DNA separation), annealing and extension elongation) et 2020). The PCR method generally applied nowadays is real–time PCR or known as quan- titative polymerase chain reaction (qPCR). It has advantages compared to conventional PCR (cPCR) that can continuously record the products accumulation during the cycle where cPCR still relies on agarose gel electrophoresis to determine the amplicons (Dorlass et al. 2020).

The quantity of qPCR is calculated by applying the threshold cycle (Ct) based on fluorescent intensity induced by noisuoresoises the attachment of DNA solution isolates

along with PCR reagents in the tube wall (Karami et al. 2020). Several advantages of qPCR have been studied and published by researchers (Yang et al. 2020; Cellier et al. 2020; Guo & Pooler 2020; Ahmed et al. 2020; Kim et al. 2020; Farhan et al. 2020; Zheng et al. 2020) owing to the high preci - sion and accuracy during the detection in each cycle (exponential phase) compared to cPCR that determines in the final phase of amplification (the plateau phase) due to the accuracy is lower than qPCR (Karimi et al. 2020).

Ferreira et al. (2018) studied the assessment of conventional PCR and real-time PCR for screening Streptococcus aga- lactiae in pregnant women, and the result shown among the 130 clinical specimens used in the study. In comparison, 23 (17.7%) of the clinical specimens tested positive for GBS colonization with conventional PCR, and 38 (29.2%) tested positive with qPCR. The study concluded that the qPCR technique had a better performance in identifying positive SGB clinical specimens than conventional PCR. The basic of PCR technique is the selection of primer to be used.

The specific primer will be at- tached to the region-specific to the DNA template and amplified into a new strand. A precise primer design is required to produce specific primers that match the target amplification. To detect porcine and bovine DNA, one of the genes that can be Cytochrome b gene (cyt b). Cytochrome b (mt Cytb) gene has been as efficient with power characterization both and science (Saif et al., 2012). It is also used in stud- ies of molecular evolution (Prusak et al., 2004). The gene length is 1140 bp and has some stable sequences used to suggest universal primers for typical PCR-based methods (Parson et al., 2000).

Method validation is the practical process of determining the suitability of a method for pro- viding data is for intended purpose. For any method to produce meaningful and reliable data, some performances checks should be made before the method is applied to a real sample (Ali et al., 2012). There are many performances characteristics that can potentially be investigated for a particular method, some of which are used in this study.

Specificity is the ability to measure only cer - tain substances carefully and thoroughly with the other present the matrix (Brown, 2005). The component of the specificity test in this study is the oligonucleotide primer. The nucleotide sequence is specified using NCBI Blast software to confirm the species origin of the sequence of oligonucleotide primer. The study determined the detection limit (DL) of each PCR method under their optimal DNA. Practically there are several ways of de- termining the detection limit of a method. The analyte is typically diluted serially in qualitative Comparison of Real-Time ... | Ahlam I., Annisa F., Emelda, Muhammad Abdurahman M.

| 65 analysis until it can no longer be detected reliably information to show how accurate and reliable real-time PCR compare to conventional PCR. The study compares both assays using a descrip- tive The techniques both assays make it is impossible to compare quanti - tatively. This study aimed to introduce a suitable and sensitive technique between real-time and conventional PCR. 2. EXPERIMENTAL SECTION 2.1 Materials and Method 2.1.1

Sample Preparation samples from porcine and bovine sources were obtained Eurofins, (Table Porcine and bovine DNA were prepared by dis- solving the control DNAs of porcine and bovine in distilled water with series of concentrations 10-1 - 10-5 ng/μl. Table 1. General description of control DNAs em- ployed in this study Genomic Type Company Name Batch no. Con- centra- tion Genomic DNA of Cattle (Bos taurus) Eurofins 150 [ng Genomic DNA of Pig (Sus scrofa domes- tica) Eurofins 5212581501 150 [1 / μl] 2.1.2 Primer Design A DNA sequence of the mitochondrial genome was obtained from Tanabe et al. (2007).

Regions with high similarity were chosen for primer bind- ing sites in the area coding for the Cytochrome b gene. The specificity all was checked with the Primer-BLAST software (Basic Local Alignment Search Tool, NCBI). The of porcine genomic DNA were 5′ -CTT GCA AAT CCT AAC AGG CCT G -3′ (forward) and 5′ -CGT TTG CAT GTA GAT AGC GAA TAA C-3′ (reverse). primers for amplification bovine DNA 5′ -CCC GAT TCT TCG CTT TCC AT-3′ (forward) and 5′ -CTA CGT CTG AGG AAA TTC CTG TTG-3′ (reverse). Custom synthetic oligonucle - otide primers were obtained from IDT.

The sizes were small (131 bp and 120 bp, respectively), degradation possible in highly processed prod- ucts. 2.1.3 Real-Time PCR Assay Genomic DNA of cattle and pig were diluted and subjected to the SYBR green-based PCR. The reaction was carried out using the SsoAdvanced USA) 10.4 μL, with the 10 μM and 0.4 μL of reverse and forward primer, and a DNA concen- tration to μL. The kit a reagent to help the PCR assay to send a signal and stabilize the PCR assay. The kit contains dNTPs, MgCl 2, SYBR green I dye, enhancers and Amplification performed the StepOnePlus System (Applied Biosystems, USA) under the following conditions of tempera - ture cycling: initial at ?C 10 minutes (pre denaturation stage); 40 cycles at 95 ?C for 10s (denaturation stage), and continued at 63 for (annealing elongation The stage was continued to measure melting temperature 1 at ?C 15s then cooled 60 for s. stage the fluorescence signal at the end of each cycle.

The results were analyzed using the cycle threshold (Ct) and Tm. 2.1.4 Conventional PCR assay This study used the same reaction as described performed in the T100 Thermal Cycler (BioRad, USA). The performance was begun at 95 oC for 7 minutes and continued

with the denaturation stage at a similar temperature for 30 seconds. The second stage was the annealing stage, where primer was designed to anneal the single-stranded
 DNA target.

While for porcine primer, the an- nealing stage was at 63oC whereas the annealing stage for bovine primer was at 61 oC. The stage was repeated for 40 cycles. Furthermore, the elongation was the third step that must be oc - curred at temperature 72oC and proceeded to the last step at a similar temperature for 7 minutes. Afterward, the PCR products were determined using in agarose in TAE followed gel staining visualization under UV light transillumination. The 1 kb DNA ladder marker was applied to determine the size of all DNA fragments. 2.1.5

Statistical Analysis performance is by constructing a standard curve from a serial dilution template (Hofmann et al., 1999). type-I (a) 5% equivalent 95% coverage for genomic DNA was used for all analyses. Correlation between Ct-values against the log of the target concentration was calculated using Pearson's correlation coefficient and as associated 2 (which is the squared correlation, the percentage of variance calculated according to the equation: 10 (-1/slope) – 1. The calculation was performed in Microsoft Excel (Redmond, 3. RESULTS AND DISCUSSION 3.1 Real-time PCR The diagnostic status of the sample was deter - mined based on the obtained Ct value.

The range of Ct values for SYBR Green dye in porcine samples was 18.14 – 31.94. While the bovine sample was 19.84 – 34.46. The Ct values range was exceeding than and may stated that the Ct values obtained from the two primers are acceptable (Table 2). Five-fold dilution series of 10 -1 –10 -5 gave stan- dard curves for detecting the genomic DNA of each bovine and porcine in real-time PCR. The one-step PCR efficiency R2 values were 97.4% and 0.962 for porcine, and 90.6% and 0.995 for bovine, respectively (Figure 1). The correlation between the Ct value and the concentration five-fold using Pearson's analysis showed a negative correlation (Porcine: -3.386 and Bovine: -3.569) with a p value <0.05 (Figure 1), indicating the higher the concentration of DNA in the DNA sample, the lower the Ct value obtained. On the other hand, the lower the DNA concentration, the higher the Ct value. Figure 1.

Standard Curves of 5-fold Dilutions of Por - cine and Bovine DNA The real-time quantitative PCR method proposed in this study allowed us to detect each each DNA species was clearly observed in a range between 0.1-0.00001 ng/µl. In the case of 0.00001 of each DNA species, amplification was apparently detected. Hence, we concluded that the limit of detection of those porcine DNA and bovine DNA species were10 -5 ng/µl since it has shown an amplification curve for this concentra - tion. For

the primer specificity test for bovine and which can be seen in Figure 2 for the porcine Table 2. Mean Ct Values Obtained with the Real- time PCR Con- cen- tration (ng/ µl) Porcine Primer Bovine Primer Mean ± SD % RSD Mean ± SD % RSD 10 -1 18.14 0.79 3.99 19.84 1.58 8.69 10 -2 21.92 1.07 4.60 23.22 2.13 9.70 10 -3 24.77 1.31 4.90 26.79 1.14 4.60 10 -4 28.11 2.07 7.12 29.02 0.37 1.31 10 -5 31.94 0.66 1.90 34.46 0.75 2.36 SD: standard deviation; RSD: relative standard deviation Comparison of Real-Time ... | Ahlam I., Annisa F., Emelda, Muhammad Abdurahman M. | 67 test and Figure 3 for the bovine test where the increase the curve.

shows that the SYBR Green method with bovine and pig primers can amplify their respective DNA amplification process using SYBR Green can be analyzed Melting specific - plification process will produce one type of peak with the same Tm value (Figure 2 and Figure 3). However, in porcine DNA testing, a Tm peak ap- peared, which was known to come from a nega- tive control sample (bovine DNA) at 79 oC that indicated that non-specific amplification products had been formed. The event is often referred to as mispriming or primer-dimer. Primer-dimer is the formation of a secondary structure caused by the annealing of similar primers or dissimilar prim- ers, such as between forward primers and reverse primer complements.

Meanwhile, mispriming is the attachment of primers outside the target DNA sequence (Ponchel, 2007). (a) (b) Figure 2 . The amplification plot of Porcine DNA using qPCR (a) amplification plot and (b) Melting Curve (a) (b) Figure 3. The amplification plot of Porcine DNA using qPCR (a) amplification plot and (b) Melting Curve Comparison of Real-Time ... | Ahlam I., Annisa F., Emelda, Muhammad Abdurahman M. | 68 3.2 Conventional PCR The of amplification conven- tional PCR were described in gel documentation (Figure 4) and showed that the genomic bands in bovine and porcine DNA were clearly visible without smear.

It can be concluded the DNA of cows and pigs has a high purity in low concentra - tions. was out - being tested. The gel documentation was carried out for DNA due the insufficient of in gel in one process. conventional PCR was shown in Figures 4a and 4b. Figure it be that primers for bovine can only amplify DNA se- quences in bovine species and cannot amplify DNA sequences in pig species (Lane 11, 12 and 13). results obtained porcine primers in 4b occurs on lanes 11 and 12 (Bovine DNA). Improper annealing temperature can cause DNA not to be amplified miss-priming amplification.

Thus, of primers tested at annealing temperatures of 60, 61 and 62 oC and reduced the number of PCR cycles to 30. However, the same result is seen on gel. The porcine can specifically the porcine DNA. (b) (a) Figure 4. Visualization of conventional PCR of (a) bovine and (b) porcine. Gel analysis of the Conventional PCR products of 5-fold dilutions

of bovine and porcine DNA to determine sensitivity and specificity. Lane M, Ampli- Size 300-10,000 base pairs (bp) in 1 Kb increments. Lane 1-2: 0.1 ng/µL: Lane 3-4: 0.01 ng/ µL; Lane 5-6: 0.001 ng/ µL; Lane 7-8: 0.0001 ng/ µL; Lanes 9-10: 0.00001 ng/ µL; Lane 1 1-13: negative control; Lanes 14: blank.

Comparison of Real-Time ... | Ahlam I., Annisa F., Emelda, Muhammad Abdurahman M. | 69 Although in sensitivity test porcine primers can detect porcine DNA in the concentration of 10-5 ng/20 µl (Figure 4b), while bovine primers are only sensitive to the presence of bovine DNA to concentration of 10 -4 ng / 20 µl (Figure 4a), The porcine primer designed by Tanabe (2007) was not effective and efficient to identify porcine DNA due to the lack of specific porcine detection. 4.

CONCLUSION According to the application of qPCR and cPCR, both showed satisfactory sensitivity during the analysis of porcine and bovine genomic DNA. concentration (10 -4 - 10 -5 ng/20 µl). Unfortu- conventional PCR still requires optimization of annealing temperature. Optimization of annealing temperature is one of the important parameter criteria for the success of PCR. While each PCR method its and a choice the PCR method depends on the purpose of its species product. 5.

CONFLICTS OF INTEREST The authors declare that they have no known competing financial interests or personal relation- ships could appeared influence work reported in this paper. 6. ACKNOWLEDGEMENT The authors wish to thank the Faculty of Science Technology, Universiti Sains Islam Malaysia (USIM) providing facilities finish study.

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