Alternative Quantitative Digital Analysis of Agarose Gel PCR Products for Detection of Molecular Markers in Livestock

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ABSTRAK

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Metode Polymerase Chain Reaction (PCR) konvensional sudah menjadi prasyarat dalam penelitian biologi molekuler. Reaksi PCR mudah disusun dan hanya membutuhkan sebagian kecil dari urutan nukleotida target yang kompleks, sehingga PCR menjadi metode yang mudah dan akurat untuk digunakan dalam analisis biokimia dan molekuler. PCR secara umum dibedakan menjadi dua yaitu PCR kualitatif dan real-time PCR kuantitatif (RT-qPCR). Metode RT-qPCR lebih presisi namun memiliki kelemahan yaitu biayanya jauh lebih mahal dan membutuhkan peralatan lebih rumit dibanding PCR konvesional. Produk PCR divisualisasikan menggunakan elektroforesis gel agarose yang menghasikan pita. Seiring dengan perkembangan teknologi digital, pita yang dihasilkan dapat dianalisis dengan menggunakan sowftware digital yang biasa digunakan untuk menganalisis foto seperti ImageJ dari NIH. Hasil uji coba menggunakan perangkat lunak ImageJ untuk menganalisis CD44 dengan gen housekeeping β-Actin menunjukkan bahwa ekspresi gen dapat diekspresikan secara kuantitatif. Ekspresi kuantitatif CD44 dan β-Actin diperoleh dengan membandingkan persentase plot puncak CD44 dan β-Actin. Ekspresi CD44 lebih tinggi daripada β-Actin setelah dianalisis menggunakan perangkat lunak ImageJ. Hasil ini juga konsisten dengan hasil RT-qPCR yang membutuhkan peralatan dan reagen PCR yang lebih kompleks. Metode analisis hasil PCR semi kuantitatif menggunakan perangkat lunak ImageJ dapat menjadi alternatif bagi laboratorium peternakan dan veteriner yang memiliki keterbatasan anggaran penelitian dan peralatan.

Kata Kunci: Kuantifikasi Ekspresi Gen, ImageJ Software, Biologi Molekuler, RT-PCR, Laboratorium Veteriner

ABSTRACT

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The conventional polymerase chain reaction (PCR) method has become a prerequisite in molecular biology research. The PCR reaction is easy to prepare and only requires a small portion of the complex target nucleotide sequence, making PCR an easy and accurate method to use in biochemical and molecular analysis. PCR is generally divided into two categories: qualitative PCR and quantitative real-time PCR (RT-qPCR). The RT-qPCR method is more precise but has the disadvantage that it is much more expensive and requires more complicated equipment than conventional PCR. PCR products were visualized using agarose gel electrophoresis, which produced bands. Along with the development of digital technology, the resulting bands can be analyzed using digital software commonly used to analyze photos, such as ImageJ from the NIH. The trial results using ImageJ software to analyze CD44 compared to the housekeeping gene β-Actin demonstrated that gene expression can be quantified. Quantitative CD44 and β-Actin expression measurements were obtained by comparing the percentage of their respective peak plots. Analysis showed that CD44 expression was higher than β-Actin when evaluated with ImageJ software. These findings align with RT-qPCR results, which require more advanced PCR equipment and reagents. The semi-quantitative PCR analysis method using ImageJ offers a practical alternative for livestock and veterinary laboratories with limited budgets and resources.

Key Words: Gene Expression Quantification, ImageJ Software, Molecular Biology, RT-PCR, Veterinary Laboratories

INTRODUCTION

Genetic material consists of DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid), which are substances that control all body activities. The role of DNA and RNA is what encourages researchers to develop methods of detecting genetic material for various purposes. In 1983, a biochemist named Kary Mullis discovered the Polymerase Chain Reaction (PCR) technique, which became the basis of modern PCR that is developing today (Bartlett et al. 2003). PCR is a technique of multiplying a small number of specific nucleotide sequences in the DNA of a complex organism in vitro so that it is ready for analysis (Kubista et al. 2006). The PCR reaction is easy to set up. It only requires a small portion of the target nucleotide sequence, making PCR an easy, cheap, and accurate method for biochemical and molecular analysis (Green

& Sambrook 2019). The PCR method has been widely used in forensic analysis (Morling 2009; Gibson-Daw et al. 2018), food technology (De Medici et al. 2015; Chapela et al. 2015), medical diagnostics (Zauli 2020; Ai et al. 2020), livestock and veterinary diseases (Hewajuli et al. 2014; Hamond et al. 2014; Knapp et al. 2014; Kishimoto et al. 2017) and various research interests in the field of molecular biology. PCR has also become popular since it was used as a standard test for diagnosing SARS-CoV-2, the cause of COVID-19, which was designated by WHO as a pandemic (Waller et al. 2020; Zhu et al. 2020).

PCR is generally divided into two categories: qualitative PCR and quantitative real-time PCR (RT-qPCR). In principle, qualitative and quantitative PCR are the same process; the only difference is the interpretation of the results. In qualitative PCR, only positive or negative results, as indicated by bands on the agarose gel electrophoresis, result when read on a UV transilluminator. RT-qPCR shows positive or negative results and can also show how much DNA or genes are present in the sample (Garibyan and Avashia, 2013). Various quantitative parameters make the RT-qPCR method more precise (Kralik & Ricchi 2017). However, RT-qPCR has the disadvantage that it is much more expensive than conventional PCR and requires more complicated equipment than conventional PCR.

The results of the analysis of PCR products using agarose gel electrophoresis when read in a UV-transilluminator are in the form of a band image of the target gene. The strength and weakness of the resulting band indicate the target gene's expression. Therefore, along with the development of digital technology, there is potential to analyze the bands produced using digital software commonly used to analyze photos, such as ImageJ (Rueden et al. 2017). However, there are still very few references to using this software, especially in Indonesia's veterinary and livestock fields. This article will discuss and demonstrate the use of ImageJ software for gene expression analysis of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) results. This method can be an alternative for laboratories in developing countries with limited research budgets, especially livestock and veterinary laboratories.

MATERIALS AND METHODS

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

According to the manufacturer's instructions, RNA was isolated from ovine testicular cells using Agilent RNA Isolation Kits (Agilent, USA). All reagent preparations are set on ice. Reverse transcriptase reactions were performed by adding ReverTra Ace (Toyobo, Japan) to the RNA solution. The mixture was incubated for 10 minutes at 30°C, 60 at 42°C, and 5 at 99°. Standard PCR reactions will be performed at 1000 ng cDNA per 20 µL PCR reaction mixture. PCR products will be separated and visualized on a 2% (b/v) agarose gel containing ethidium bromide. The visualization results of PCR products in agarose gel are then analyzed quantitatively using ImageJ software (NIH).

Visualization of PCR products in agarose gel using ImageJ Software (NIH)

Image-J software to analyze PCR results begins with downloading ImageJ software from NIH that is compatible with the computer operating system. Extract the zip file and find the ImageJ image icon in the folder. Import the agarose gel image to be analyzed by opening the file menu and selecting the file in the saved folder; the image must be converted into an 8-bit image first. The next step is to reduce the background or noise in the agarose gel image, the yellow arrow points to the background or noise that needs to be removed before quantification. How to reduce background or noise by clicking on the rectangle tool and selecting the background or noise to be removed, then clicking analyze and set measurements, then selecting a mean gray value, all and all other options can be deselected, and then clicking okay. Again, click the analyze and measure dialog box, showing the mean gray value of the background or noise. Select the yellow rectangle box from the agarose gel image, click process math, subtract, enter the mean gray value of the background or noise, and click OK. The next step is to invert the image so that the gel band in the image appears black on a white background. Select the rectangle tool again, then draw a rectangle on the gel band. Click analyze gel, select the first lane dialog box, and confirm if the bands are arranged horizontally. Click yes to continue if the gel band is in the horizontal position. Now, the rectangle box on the gel is marked as one. Again, click analyze gel and plot lanes. Next, select the line tool and draw a straight line connecting the bottom ends of the plotted lanes. This step displays the area occupied by the bands on the gel image. Once done, click inside the plot. Again, click on analyze gel and label peaks; this step displays the percentage intensity of the gel, then copy the entire result and paste it into an Excel file. Next, repeat the previous procedure for β-Actin gene housekeeping in the same way to calculate the intensity of the percentage area. After the results are obtained, copy and paste the entire data into the Excel file again. To measure the normalized intensity of the target gene gel band against the housekeeping gene, use the formula of the percentage intensity of the area obtained by the target gene divided by the percentage of the housekeeping gene, and then the resulting value can be plotted on a graph so that the

densitometric analysis of the agarose gel band has been completed.

Quantitative Real-Time PCR (RT-qPCR)

Complementary DNA (cDNA) from the previous RT-PCR reaction was then used in quantitative PCR (RT-qPCR) to determine CD44 gene expression levels on the Rotor-Gene Q (Qiagen, USA), utilizing the SsoFast EvaGreen Supermix (Biorad, USA). The sequence of the β-Actin is as follows: Forward: 5'-TCCCTGGAGAAGAGCTACGA -' 3 and Reverse: 5'-ACATCTGCTGGAAGGTGGAC -3'. The sequence of the CD44 is as follows: Forward: 5'-CGGATACCAGAGACTACGGC -'3 and Reverse: 5'-CCGCATAGGACCTGAGGTTG -3'. The RT-PCR master mix was made with a total volume of 20 µL with the following composition: SsoFast EvaGreen Supermixes (10 µL), 10 µM forward primer (0.4 µL), 10 µM reverse primer (0.4 µL), cDNA template (2 µL) and Nuclease Free Water (7.2 µL). A two-step amplification program was used with pre-denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 5 seconds) and annealing (58°C for 15 seconds). The amplification of both the target and reference genes was performed in triplicate. The relative quantification of target gene mRNA was determined using the comparative CT method based on the difference in cycle threshold (ΔCT) values between the target and reference genes.

RESULTS AND DISCUSSION

Conventional polymerase chain reaction (PCR) should be a prerequisite in molecular biology research. However, the data obtained can only be interpreted as "positive" (detectable) or "negative (not detectable)". Conventional PCR is an endpoint assay where the amplified PCR product (amplicons) can only be detected at the end of electrophoresis using ethidium bromide (EtBr) and other nucleic acid dyes. Quantitative PCR (qPCR) is a more sensitive assay. However, qPCR is still not a standard test tool in most veterinary laboratories and, most importantly, in less developed countries where access to real-time PCR technology is limited or non-existent.

PCR visualization on an agarose gel

Figure 1 a) shows the visualization of one of the bands on the agarose gel with background/noise in the green box. This background/noise can be removed by performing the "subtract" step described in the materials and methods. Meanwhile, Figures 1.b) and 1.c) show the results of the subtract and invert image process using ImageJ software from the expression of the CD44 gene

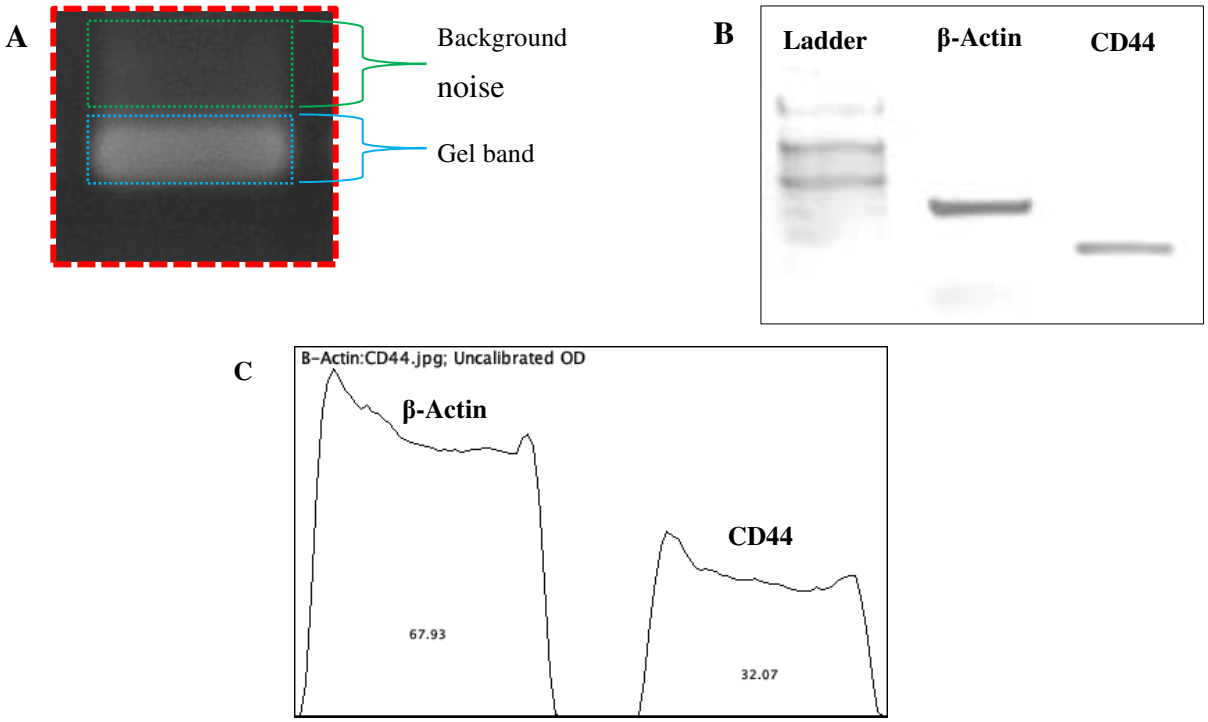

Figure 1. Visualization of RT-PCR results on agarose gel: a) gel band image and the resulting background/noise; b) the result of the subtract and invert image process using ImageJ software from gene expression of CD44 as the target gene and the β-Actin gene as the housekeeping gene; c) plot peak of optical density with Image-J software to determine the strength and weakness of gene expression from RT-PCR results of agarose gel electrophoresis

as the target gene and the β-Actin gene as the housekeeping gene. Visually, the expression of the CD44 gene in Figure 1.b shows that sample 2 has more thickness than the other samples.

In general, the PCR stage starts with the denaturation of DNA templates by heat, annealing, and extension, which repeatedly occurs in an enzymatic reaction with a DNA polymerase catalyst (Green & Sambrook 2018). The results of this PCR process need to be analyzed further to be interpreted. PCR products can generally be detected by two methods, including: 1) staining PCR products with chemical dyes such as ethidium bromide or 2) labeling PCR primers or nucleotides with fluorescent dyes (fluorophores) before PCR amplification (Garibyan & Avashia 2013). After staining, agarose gel electrophoresis is the simplest and easiest method to visualize and analyze PCR products. Electrophoresis separates charged molecules in an electric field (Fatchiyah et al. 2011). With this method, PCR products are distinguished based on their size, which is then compared with the visualization of the control (ladder).

Conventional PCR tests are now available in most veterinary pathology laboratories. However, the data obtained can only be interpreted as "positive" or "negative (not detected)." With the development of digital technology, there is potential to analyze the bands generated using digital software commonly used to analyze photographs, such as ImageJ (Rueden et al. 2017). Some other image processing tools include Matlab (Fan & Quake 2007; Zhu et al. 2014; Dimov et al. 2014) and LabView (Zhong et al. 2011).

Densitometric analysis of agarose gel electrophoresis results using ImageJ

ImageJ is a pioneer software for scientific data analysis in the form of images developed by the US National Health Institute (NIH) since 1987 (Schneider et al. 2012). ImageJ has been widely used for scientific data analysis, such as PCR (Lee & Back 2017; Chung et al. 2019), immunocytochemical staining (Das et al. 2014; Lee et al. 2015), immunoblotting (Jiao et al. 2014; Gallo-Oller et al. 2018), and colony counting in cell culture (Guzmán et al. 2014; Choudhry 2016). ImageJ provides an alternative low-cost PCR solution, especially for developing countries with limited RT-qPCR equipment. In this paper, the use of ImageJ software to analyze electrophoresis images has been demonstrated. The target gene analyzed was CD44 with the housekeeping gene β-Actin. The CD44 gene encodes a cell-surface glycoprotein pivotal in numerous biological processes, including cell adhesion, migration, proliferation, and signaling (Laohavisudhi et al. 2022). CD44 plays crucial roles in immune responses, wound healing, and cancer biology, often overexpressed in tumors and contributing to cancer stem cell maintenance, metastasis, and drug resistance(Laohavisudhi et al. 2022; Shen et al. 2022). The dysregulation of CD44 is linked to various diseases, including rheumatoid arthritis, cancer, and infectious diseases. CD44 serves as a valuable biomarker for cancer prognosis and a potential therapeutic target in oncology and autoimmune conditions. The activation of the CD44 receptor with its major ligand hyaluronan has been shown to promote breast cancer metastasis to the liver (Ahmad et al. 2023). The β-Actin gene encodes β-Actin, a key cytoskeletal protein that maintains cell structure, motility, and intracellular transport. In mammals, β-Actin is highly conserved and ubiquitously expressed in almost all cell types, playing a critical role in processes such as cell division, signaling, and adhesion. Due to its stable and consistent expression under normal conditions, β-Actin is widely used as a housekeeping gene in molecular biology experiments such as quantitative PCR and Western blot (Bustin 2000).

The rapid development of diseases affecting the veterinary community, both animals and humans, requires accurate and rapid diagnostic methods. RT-qPCR has become a standard method of diagnosis in veterinary laboratories (Toohey-Kurth et al. 2020). The method described in this study is not intended to replace the qPCR method but to be an alternative to quantitative assessment of low-cost PCR products, especially for veterinary laboratories or livestock laboratories that have limited facilities and budgets. This method can also be used as an early indicator of possible trends that may exist across the evaluated specimens to make quick empirical decisions on the choice of specimens for further PCR testing. With the growing number of emerging pathogens, some of which are zoonotic, this method can also be used as an initial identification for faster and more precise follow-up testing decisions on possible new disease outbreaks that will develop either in animals or, if possible, zoonotic.

Table 1. Relative expression of CD44 to B-Actin as determined by optical density (OD) Image-J software

No	Genes	Area (OD)	Percent
1	B-Actin	34252.85	67.93
2	CD44	16171.18	32.07
Relative expression			0.47

Table 2. Expression level of CD44 as determined by RT-qPCR

No.	Sample	β-Actin	CD44	ΔCt CD44
1.	Sample T (control)			
	R1	18.23	28.71	10.48
	R2	18.04	28.59	10.55
	R3	20.27	28.37	8.10
	Average ΔCt CD44 T (control)			9.71
2.	Sample P1			
	R1	15.37	23.56	8.19
	R2	15.57	20.94	5.37
	R3	16.65	21.89	5.24
	Average ΔCt CD44 P1			6.27
	Fold Difference			10.88

Quantitative analysis of CD44 gene expression using RT-qPCR

Quantitative Real-Time PCR (qRT-PCR) is a powerful technique that allows researchers to quantify gene expression levels in biological samples accurately. This study focuses on the analysis of the CD44 gene, with the β-Actin gene serving as the housekeeping gene for normalization. The analysis begins by measuring each sample's Ct (cycle threshold) values for CD44 and β-Actin. The difference between these Ct values, known as ΔCt, is then calculated to determine the relative expression of CD44 within each sample (Garcia and Ma 2005). To assess changes in CD44 expression between samples or conditions by comparing the ΔCt value of the target sample to that of a control sample, resulting in the ΔΔCt value. The final step involves calculating the relative fold change in expression using the formula 2^(-ΔΔCt) (Garcia & Ma 2005; Kishore et al. 2013; Arya et al. 2017).

The Cycle Threshold (Ct) value indicates the cycles at which the fluorescence signal amplification exceeds a certain threshold to be considered significant (Kubista et al. 2006; Hahn & Lapaire 2013; Kralik and Ricchi 2017). In real-time PCR (qPCR), the Ct value determines the initial target DNA or RNA amount in a sample. A low Ct indicates that the target DNA or RNA is present in large quantities at the beginning, so the signal is detected more quickly, while a high Ct indicates a low amount of target because it requires more cycles to reach the threshold of detection (Kubista et al. 2006; Forootan et al. 2017). The data presented in Table 2 shows that the Ct value of β-Actin is consistently lower than that of CD44 across all samples; this indicates that β-Actin expression is higher than CD44, aligning with the results obtained through ImageJ analysis. Further analysis using ΔCt and ΔΔCt reveals that the relative fold change in CD44 expression between the control and sample P1 is 10.88.

CONCLUSION

The trial results using ImageJ software to analyze CD44 compared to the housekeeping gene β-Actin demonstrated that gene expression can be quantified. Quantitative CD44 and β-Actin expression measurements were obtained by comparing the percentage of their respective peak plots. Analysis showed that CD44 expression was higher than β-Actin when evaluated with ImageJ software. These findings align with RT-qPCR results, which require more advanced PCR equipment and reagents. The semi-quantitative PCR analysis method using ImageJ offers a practical alternative for livestock and veterinary laboratories with limited budgets and resources.

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