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Detection of *Rusa spp.* DNA in ready-to-eat food (RTF) using singleplex of Polymerase Chain Reaction

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Abstract

Deer meat is more expensive than meat from domesticated animals, making it a possible target for adulteration. As a result, a practical approach for detecting deer DNA was developed by employing a Polymerase Chain Reaction (PCR) assay to target a region of the mitochondrial Cytochrome Oxidase Subunit I (COI) gene in locally ready-to-eat food (RTE) products. A specificity trial was performed on eight deer-based RTF to include *Kerutup Rusa, Kari Tulang Rusa, Kurma Rusa,* and *Rendang Tok Rusa* food matrices (to include the four commercially processed RTE food products) and deer DNA was detected from four commercial RTE products (*Kerutup Rusa, Kari Tulang Rusa, Kurma Rusa* and *Rendang Tok Rusa*) with the CerV primers at 0.0001 ng/µL detection limit, showing that both the primers and the assays were effective at detecting DNA targets in thermally treated matrices. This assay addresses critical gaps in halal certification and food fraud prevention, offering a reliable tool for regulatory enforcement and consumer protection in Malaysia's growing RTE market. The CerV gene detection limit was 0.0001 ng of CRM, consistent with the Certified Reference Material (CRM) used in this investigation (Red Deer). As a result of the present investigation, it is clear that PCR targeting the CerV COI 1 gene is appropriate for identifying deer DNA in RTE food samples.

1. Introduction

Consumers' eating habits and lifestyles have changed in this modern era of globalisation. Fast, ready-to-eat and frozen food are in high demand from consumers in this millennium because they are convenient. The significant changes in the processed food industry in recent years have improved knowledge of food composition and its effects on consumer health (Siro *et al.*, 2008). The food industry's continual development, which includes processing methods such as marinating, canning, or cooking, as well as the production of ready-to-eat meals, helps prevent food items from being destroyed, driving the increase in processed meat production. Consumers worldwide are now more aware of the ingredients in the food they consume, largely due to innovations in the meat production industry, which has led to increased demand for clear and accurate information labels on food products (Sentandreu & Sentandreu, 2014).

Modern methods of meat processing, such as marination, canning, and mechanical tenderisation, can mask the morphological and organoleptic properties of meat, making visual verification impossible (Flores-Munguia *et al.*, 2000). For example, a 2023 audit and report by the Malaysian Department of Islamic Development (JAKIM) stated that 15% of the 'halal-certified' meat products had species that were not reported, indicating an urgent need for DNA-based verification (JAKIM, 2023). Deer meat is sold in Malaysia for 3–5 times the price of beef and can, therefore, be highly susceptible to adulteration with cheaper meats, such as pork or buffalo. This puts consumers at risk of noncompliance regarding religious rules and health issues. All of this has led to an increase in food fraud. In situations of processed meat food fraud, the risks to customers might range from using lower-quality components to intentionally mislabeling goods. While regulatory bodies, food producers, and consumers expect high-quality control, it is critical that consumers demand greater quality control measures to avoid such fraudulent practices and protect themselves. Additionally, there is an insufficient analysis of meat authenticity, resulting in the incidence of illicit meat and unknown species in food (Bottato *et al.*, 2014).

A range of meat products, including meats, minced meats, dried meats, and pet foods, were found to have between 20% and 70% of their labels misread (Cawthorn *et al.*, 2013; Okuma & Hellberg, 2015; Quinto *et al.*, 2015). Food fraud occasionally occurs in processed meat, which has a significant impact on public health and the Malaysian economy. Customers who have meat allergies may be put in danger if there are hidden ingredients in their food. For instance, a study by Masiri *et al.* (2016) found that undeclared pork residues in beef products increased the risk of pathogen infection. Due to the impact on consumer confidence, this issue has made it difficult for the beef industry to access new

markets (Zhao *et al*., 2014).

Additionally, improper meat product labelling results in the illegal sale of endangered, protected species like deer and thwarts efforts to conserve these animal species. Therefore, commercial food products must not raise health concerns for consumers to comply with national and international food rules (Ballin, 2010). Food manufacturers are prohibited from making claims about the composition, quality, origin, or processing of food products that are intentionally false or deceptive, as food products must also be legitimate. On the other hand, food adulteration has become an international issue. According to studies, meat products are frequently tampered with (Premanandh *et al*., 2013; Fajardo *et al*., 2010).

In general, DNA identification techniques have changed and revolutionised methods used for the conservation of deer populations around the world. This knowledge is valuable when formulating management approaches to protect threatened or endangered species. One of the famous techniques in molecular biology is named PCR (polymerase chain reaction), which involves the amplification of a target DNA sequence.

Now, beyond the forensic context, PCR methods for identifying deer DNA are fundamental to deer conservation efforts. These methods help researchers understand how deer populations change, where deer genetic diversity is located, and how well conservation strategies are effective. This makes PCR very accurate for identifying deer species within populations, which provides researchers with information about where deer populations are, how many there are, and how variable their genetic makeup is. In addition, these methods may be useful for identifying hybridisation between deer species and detecting threats to the genetic integrity of these species.

This method enables researchers to explore and analyse genetic diversity and structure within deer populations. The amplification process through PCR is essential for accurately identifying deer DNA. A notable variant of this technique is qPCR, or quantitative polymerase chain reaction, which offers several advantages over conventional light microscopy methods for identifying deer DNA. PCR has sensitivity, rapidity and a good chance for future automation (McLennan *et al*., 2021). Researchers can use PCR to get high specificity for the amplification of only the DNA of the species they want to study. Importantly, PCR provides a method for quantifying deer DNA, which enables the monitoring of gene flow in deer as well as changes in the genetics of deer populations. qPCR has such high sensitivity that it can detect small amounts of deer DNA.

This method enables the simultaneous amplification of multiple DNA targets, making it a suitable approach for identifying deer species. In Gaur's (2016) study, PCR amplification of eight deer-species microsatellite loci was utilised to identify different deer DNA samples. There are loci associated with deer, which help differentiate between deer species. Appropriate deer-specific primers for PCR are critical to obtaining reliably identified deer DNA. Applying CR assays from species that have been validated provides essential support for DNA samples identified as deer in a forensic capacity for poaching. Furthermore, these validated PCR methodologies can significantly contribute to conservation initiatives by facilitating the monitoring of deer populations.

The development of methods for identifying species from unidentified sources has advanced significantly due to the government of Malaysia's enforcement of biosecurity and food regulations (Armstrong & Ball, 2005; Fajardo *et al*., 2010; Bottero & Dalmasso, 2011; Ali *et al*., 2014). Such methods include quantitative PCR (qPCR), Real-Time PCR, multiplex PCR, and restriction fragment length polymorphism (RFLP) based on PCR. In order to identify animal species from various types of matrices, such as faeces, feathers, hair, saliva, skin, and urine, molecular approaches have been confirmed and well-established (Dalén *et al*., 2004; Waits, 2009).

Molecular techniques have been developed rely on the use of a common target region in mitochondrial DNA (Cytochrome Oxidase Subunit 1) with unique primers, resulting in amplicons of varying sizes in the Cervidae family (Dalén *et al*., 2004), which the primers are designed and positing well and easily discernible on agarose gels (Bottero & Dalmasso, 2011). Because mitochondrial DNA gene sequence data sets were publicly available for the target animal species (Janke *et a*l., 2002; Hassanin *et al*., 2012; Meiri *et al*., 2013; Martins *et al*., 2017), this study focused on using markers within the mitochondrial Cytochrome Oxidase Subunit I (COI). Furthermore, a specialised DNA amplification using a particular genetic marker target can assist in unambiguous identification and animal species classification based on mitochondrial cytochrome oxidase subunit I (COI). This marker has been widely adopted for DNA barcoding initiatives, where it is used for rapid and conclusive species identification. Moreover, analyses of COI I sequences can provide information on the evolutionary relationship and biodiversity of animal populations.

COI I sequences were used to accurately identify and authenticate the presence of the Rusa species in the RTF food items in this study. This method provided a reliable and effective means of ensuring that labelled products will be good and genuine. Therefore, singleplex PCR that can generate, dissociate and validate the genetic markers of the mitochondrial Cytochrome Oxidase Subunit I, as well as the commercially available CerV oligonucleotide primers, were used to detect and amplify the genetic markers of mitochondrial Cytochrome Oxidase Subunit I and Rusa spp in the Rusa labelled Ready-To-Eat (RTF) foods in Malaysia. Although PCR-based methods are available for detecting deer DNA (Druml *et al*., 2014), it is worth noting that most assays are limited to examining fresh or frozen meat. This research significantly advances the understanding of a single-plex PCR for the food matrix of processed ready-to-eat (RTE) foods, a food matrix recognised as problematic due to thermal treatment leading to DNA fragmentation (Martins *et al*., 2017).

2. Materials and methods

2.1 Sampling

Four samples that were marketed as being made from *Rusa spp*. (*Kerutup Rusa, Kari Tulang Rusa, Kurma Rusa,* and *Rendang Tok Rusa*) and one RTF made without deer meat (*Daging Lembu masak Cili Padi*) was tested. Following the manufacturer's instructions, 10 g of RTF samples were extracted using the Epicentre MasterPure™ DNA purification kit. During the RTF extraction process, blank and chicken flesh were also included, with these two indicators functioning as extraction and blank controls in the extraction method.

2.2 Extraction and amplification of the CerV gene

Genomic DNA was extracted in triplicate from 10 g homogenised samples of each RTE product (*Kerutup Rusa, Kari Tulang Rusa, Kurma Rusa, Rendang Tok Rusa,* and control *Daging Lembu masak Cili Padi*) using the Epicentre MasterPure™ DNA Purification Kit (Catalogue No. MCD85201) following the manufacturer's protocol. Blank controls (n = 3) and chicken DNA spikes (n = 3, 50 ng/µL) were included to assess extraction efficiency. DNA concentration was quantified via NanoDrop™ 2000 spectrophotometer (Thermo Fisher), and purity was confirmed (A260/A280 ratio ≥1.8). Following that, the isolated DNA was submitted to PCR amplification. CerV primers (F-5'TCT TTA TGG GCT AAC AGC-3') and (R-5'-CTT GTT CCG TTG ATC AAT T-3') were used to amplify genomic DNA targeting deer mtDNA. A volume of 1 µL of 100 ng DNA, 12.5 µL of universal PCR master mix (MyTaqTM Red Mix), 8.5 µL of sterile distilled water, and 1 µL of 25 pM each of the forward and reverse primers were used in the test (Apical Scientific SDN BHD). Amplification was carried out in a Thermal Cycler BioTone (Analytical Jena, GmbH) using a temperature program that included an initial denaturation at 95°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute. The amplified fragments were seen with a UV transilluminator after electrophoresis in 1.5 per cent (w/v) agarose 1X TBE (0.1 M Tris, 0.1 M Boric acid, 0.2 mM EDTA) at 120 V for 60 minutes (Alpha Imager TM2200).

2.3 Sensitivity studies of CerV oligonucleotide primers set

The detection limit of CerV oligonucleotide primers was investigated using Red Deer genomic DNA as Certified Reference Material (CRM). The PCR test conditions were identical to those described in PCR amplification using various oligonucleotide primers and concentrations of genomic DNA ranging from 0.0001 to 100 ng.

2.4 Reproducibility studies using the CerV gene in ready-to-eat foods (RTF) deer-based products

In the repeatability investigation, five varieties of RTF were used: *Kerutup Rusa, Kari Tulang Rusa, Kurma Rusa, Rendang Tok Rusa,* and *Daging Lembu masak Cili Padi*. The Epicentre MasterPure™ DNA purification kit was used to extract all samples, and the extraction process workflow was followed according to the manufacturer's instructions. The concentration of genomic DNA recovered was then diluted to 50 ng. Following this, PCR analysis targeting the CerV genes was performed as described earlier.

2.5 Specificity studies of CerV oligonucleotide primers set

CerV specificity experiments have been undertaken using several types of animal DNA to ensure that the virus exclusively detects deer DNA. Five (5) distinct types of animal DNA were employed in the specificity investigations, including sheep, porcine, cattle, buffalo, chicken, and red deer. The PCR analyses were carried out on the five distinct animal DNA samples using the CerV gene, as described in the earlier subheading.

3. Results and discussion

For the specificity study on an agarose gel, the amplicon generated by PCR analysis revealed a band of 116 base pairs (Figure 1). Except for the Red Deer, which revealed a positive band, none of the other animal DNA genomics (goat, chicken, wheat, soya, porcine, lamb, duck, sheep, horse, beef, buffalo, mutton) were positive for the CerV gene. The CerV primer was shown to be specific for *Rusa spp*. DNA.

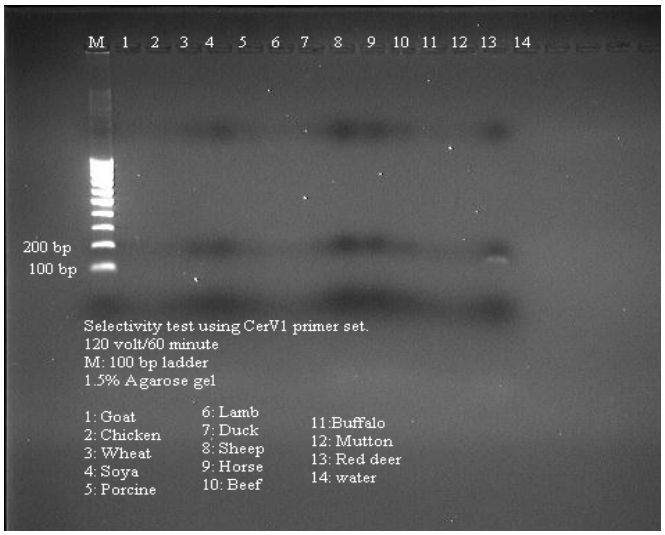

Figure 1: Specificity studies on *Rusa spp*. from genomic DNA of Red Deer. Lane M: 100 kb ladder; Lane 1: Goat DNA; Lane 2: Chicken DNA; Lane 3: Wheat DNA; Lane 4: Soya DNA; Lane 5: Porcine DNA; Lane 6: Lamb DNA; Lane 7: Duck DNA; Lane 8: Sheep DNA; Lane 9: Horse DNA; Lane 10: Beef DNA; Lane 11: Buffalo DNA; Lane 12: Mutton DNA; Lane 13: Red Deer DNA; Lane 14: Water.

The sensitivity of single-plex PCR was estimated using the target species' serially diluted DNAs (from 100 ng to 0.00001 ng per reaction). Detection limits were tested using Certified Reference Material of Red Deer DNA purchased from the manufacturer. For sensitivity, PCR analysis was used to determine the detection limit of the CerV gene using varied quantities of DNA from certified reference material, Red Deer. The lowest amounts of genomic DNA identified using the PCR test for the CerV gene were as low as 0.00001 ng/ µL (Figure 2). The high sensitivity of this assay led to the accurate and reliable detection and differentiation of meat from target deer species.

Rusa spp. DNA was detected among the four ready-to-eat foods (RTFs), which claimed to consist of deer meat (*Kerutup Rusa, Kari Tulang Rusa, Kurma Rusa, Rendang Tok Rusa*), though band eight (8) shows a smearing effect, which might be due to high DNA content (Figure 3). The following findings were obtained using a consistent forward primer/reverse primer concentration ratio (25 pmol: 25 pmol). During the experiments, no cross-reactivity with other animal species was observed. The CerV primers in this experiment could detect the genus *Rusa* genes in the RTF products. Similar findings were also reported by Khatun *et al.* (2021), who detected buffalo and chicken DNA in beef-labelled products and found buffalo DNA in cheese samples, despite the cheese being declared as bovine cheese.

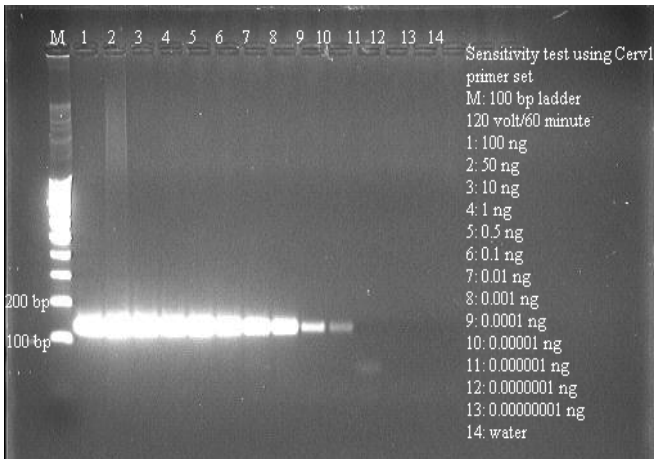

Figure 2: Detection limit of CerV gene from genomic DNA of DNA of Red Deer). Lane M: 100 kb ladder; Lane 1: 100 ng; Lane 2: 50 ng; Lane 3: 10 ng; Lane 4: 1 ng; Lane 5: 0.5 ng; Lane 6: 0.1 ng; Lane 7: 0.01 ng; Lane 8: 0.001 ng; Lane 9: 0.0001 ng; and Lane 10: 0.00001 ng; Lane 11: 0.000001 ng; Lane 12: 0.0000001 ng; Lane 13: 0.00000001 ng; Lane 14: Water.

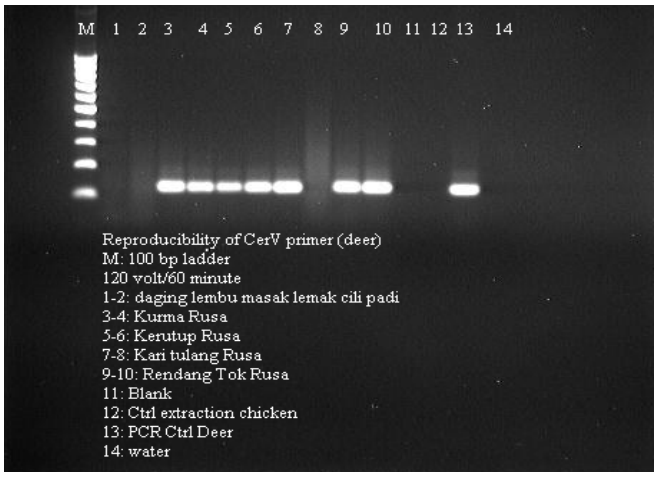

Figure 3: Detection of CerV gene in various types of ready-to-eat foods (RTFs) samples. Lane M: 100 kb ladder; Lane 1-2: *Daging Lembu masak Lemak Cili Padi*; Lane 3-4: *Kurma Rusa*, Lane 5-6: *Kerutup Rusa*; Lane 7-8: *Kari Tulang Rusa*; Lane 9-10: *Rendang Tok Rusa*; Lane 11: Blank; Lane 12: Control Extraction Chicken; Lane 13: PCR Control Deer; Lane 14: Water.

Therefore, DNA-based approaches play a crucial role in meat authenticity and may be used to detect deer meat adulteration in RTF products. Furthermore, by direct identification of *Cervus* spp. targeting particular primers in meat products, meat producers and restaurant owners could authenticate and sell their products with high confidence to the consumers (Druml *et al*., 2014; Kaltenbrunner *et al*., 2018). This current study shows that PCR analysis using CerV primer is a quick, easy, and reliable technique for detecting *Rusa spp*. DNA in food products.

As mentioned in the specificity study, the CerV primer amplified only DNA from *Rusa spp*. The PCR analysis, shown in agarose gel electrophoresis, yielded a band of 116 base pairs for the Red Deer DNA sample, confirming a positive result. In contrast, the other animal DNA genomic samples (goat, chicken, wheat, soya, porcine, lamb, duck, sheep, horse, beef, buffalo, and mutton) are negative. The results are highly specific, indicating that the assay has the capacity to clearly distinguish between deer DNA and that of any other related species. This may prove efficient in determining Rusa spp. in different food samples.

The CerV assay established a LOD (limit of detection) at 0.00001 ng/µL (Figure 2), exceeding sensitivity from previous deer-specific assays (Kaltenbrunner *et al*., 2018: LOD 0.001 ng/µL). This ultra-low LOD should provide assurance within manufactured processed foods, in which reliability can vary due to DNA degradation processes, which can reduce template availability by 90–95% (Gharst *et al*., 2013). Additionally, no cross-reactivity was observed when tested against porcine DNA (Lane 5), which is an important consideration when producing products sold in halal markets (countries with a Muslim majority).

As noted, the assay produced near-perfect sensitivity. However, a smear was noted with *Rendang Tok Rusa* (Figure 3), which could be caused by PCR inhibitors within the spices, such as turmeric, or excessive DNA loading. Future studies may want to consider utilising inhibitor-resistant polymerases or DNA clean-up techniques prior to PCR (Quinto *et al*., 2015). This high sensitivity concurs with findings suggesting that the PCR assay is capable of detecting the presence of deer DNA in very small amounts, making it useful in detecting *Rusa spp*. in ready-to-eat processed food products. This confirmed our belief that the CerV detection limit for the study was valid, as demonstrated by the CRM used, which had a detection limit of 0.0001 ng. The sensitivity and specificity of the assay to quantify deer DNA in a range of food commodities, including at such low amounts, provide confidence in detecting *Rusa spp*. in ready-to-eat products.

The reproducibility study in the current research reveals a significant function of a self-designed primer in accurately amplifying the CerV gene. This way, the authors also guaranteed that the primers provided reproducible and species-specific amplification of *Rusa spp*. DNA in any ready-to-eat food samples they would want to test. These obtained values indicate similar conditions to those of other analysed samples, suggesting that the used primers are valid and can detect deer DNA without interference from other animal species (Dalén *et al*., 2004). Additionally, the reproducibility study ensures that quantitative diagnoses can be made beyond the research setting through applications such as food analysis and forensic science, thereby refuting any argument that may be made against the specific and sensitive design of the primer. The possibility of obtaining a good agreement between the two methods reasserts the value of developed PCR assays for successfully identifying and estimating deer DNA in processed food textures (Grattarola *et al*., 2014). Together, these studies provide relatively robust experimental evidence that supports the notion that reproducibility is a critical step in the development of PCR assays, whether for food safety, forensics, or genetic conservation.

The developed assay showed that cytochrome subunit 1 (COI 1) can be used to identify deer species based on their DNA. Several studies have used COI to identify deer species and are also able to distinguish between different subspecies of deer, such as the Sika deer (*Cervus nippon*) and the Japanese deer (*Cervus nipplon centralis*) (Galimberti *et al*., 2012). Fonseca and Friend (2015) also used COI sequencing in their study to

identify the species of deer found in Portuguese markets and distinguish between different subspecies of red deer.

In conclusion, the single-plex PCR assay designed in this study effectively detects deer DNA from a range of food matrices. The results of this study also support the generalisation that COI can be used to identify deer in various types of food samples and can be useful in deer conservation and management. The developed PCR assay was equally efficient and precise in terms of recovery rate, especially for samples that had been subjected to thermal treatment, such as ready-to-eat food items. The outcomes of the study include the development of a deer-specific PCR assay optimisation that has an LOD of 0.0001 ng/µL. Additionally, the study validates the proposed assay and its suitability for identifying and measuring deer DNA in RTE foods.

The study concludes that the newly developed single-plex PCR assay for detecting deer DNA in different food matrices is effective, efficient, sensitive and specific. The assay proved valid, as the limit of detection (LOD) was 0.0001 ng/µL, and effectively identified deer DNA in ready-to-eat food products offered to traders that had been thermally treated. The study suggests that the candidate gene COI may be useful for identifying deer species, which has a positive impact on food chain traceability, providing reassurance to consumers and supporting wildlife management for the deer species. Furthermore, the validated PCR methodologies also aid in combating food misbranding and adulteration, particularly in forensic applications related to the issue of poaching (Grattarola *et al.*, 2014).

These PCR assays are useful in detecting deer DNA in ready-to-eat foods, which provides information about the composition of such foods. Therefore, validated PCR methods are beneficial because they maintain the purity of food articles concerning these components, thereby avoiding food misrepresentation or adulteration. This is crucial for ensuring the stability of the food supply and maintaining consumers' trust, as well as developing measures to protect the food system. The literature analysed prompted crucial views on the viewpoints of PCR-based techniques for identifying deer DNA. Cervini *et al.* (2006) observe that microsatellites are essential in cattle genetics and can also be used for deer species.

The validation of PCR methodologies for identifying deer DNA represents a crucial field of study with substantial ramifications for both forensic investigations and conservation efforts (Gharst *et al.*, 2013; Cervini *et al.*, 2006). It provides food safety professionals with a reliable method for identifying potential instances of contamination, thereby helping them meet FDA regulations. Furthermore, the contribution of this method can increase traceability across the food supply chains, making it easier to respond to contamination cases. The combined application of this approach with existing food safety measures may help improve consumer confidence and reduce economic losses related to foodborne illnesses. However, renewed research efforts toward optimising the sing-lex PCR technique with regard to sensitivity and specificity will enhance its application enormously in the complex food matrix. Adopting single-plex PCR methods not only enhanced the understanding of food sources and increased food consumers' confidence, but also enhanced the systems used in identifying the level of pathogens in different food crops.

The assay presented in this study is based on a single gene, and the process was designed to minimise interference from other animal species. The assay's accuracy, working range and robustness were then identified in this study. The findings are consistent with those of several authors, including Kaltenbrunner *et al.* (2018), who have developed a real-time PCR assay to identify and quantify sika deer in meat products with efficiency and reliability, meeting the established parameters for accuracy. To this end, employing these molecular techniques enables regulatory bodies to identify contamination and pinpoint its origin through genetic fingerprinting, leading to the implementation of the aforementioned approaches. Moreover, due to the enhanced sensitisation to food pathogens, there is a need to understand the food labels used. Real-time PCR applications are best suited to address this issue, enabling consumers to verify the truth in claims regarding the species of meat involved or the presence of allergens in processed foodstuffs. Thus, the progress towards increased accountability may lead to increased stringency of rules and norms within the industry, a healthy focus on food safety, and proper encouragement of innovation.

4. Conclusion

Overall, the study demonstrates that the developed singleplex PCR assay is easy to use, efficient, sensitive, and specific for detecting *Rusa spp.* DNA in a variety of food matrices with a limit of detection (LOD) of 0.0001 ng/µL. This assay may serve as a valuable method for monitoring food fraud in food products containing deer meat. This PCR assay is helpful in verifying food authenticity, such as detecting mislabeling and adulteration. The assay can also help trace the origin of deer meat in food products, thereby maintaining transparency in the food supply chain. The developed PCR assay is a promising tool for regulatory agencies and food manufacturers to enforce labelling regulations and assure consumer trust due to its high sensitivity and specificity. In conclusion, the PCR assay offers a reliable method for detecting deer meat in a wide range of food products, thereby enhancing consumer trust and compliance within the food industry.

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