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**Research article**

## PCR-based identification of animal species in commercial sausage products using cytochrome b gene markers

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**Abstract**

Background and Aim. Authentication of meat species in processed products is important for food quality control and consumer protection. This study evaluated the applicability of a PCR assay targeting the mitochondrial cytochrome b (*Cyt b*) gene for species identification in commercial sausage products.

Materials and Methods. Reference samples of horse, cattle, sheep, pig, and chicken meat were analyzed together with 15 commercial sausage products. Total DNA was extracted and amplified using species-specific *Cyt b* primers. PCR products were analyzed by agarose gel electrophoresis and selected amplicons were confirmed by sequencing.

Results. Species-specific amplification products corresponding to the expected fragment sizes were obtained for all investigated animal species. Analysis of commercial sausage products revealed both agreement and discrepancies between declared and detected meat species composition. Sequencing confirmed the identity of selected PCR products.

Conclusion. The *Cyt b*-based PCR assay proved suitable for qualitative identification of animal species in thermally processed meat products and may be useful for routine authenticity testing of meat products.

**Keywords:** meat adulteration; *Cyt b;* PCR; food control; meat species identification.

### Introduction

Meat adulteration through the substitution of high-value meat species with less expensive alternatives remains a significant concern for food safety, consumer protection, and regulatory authorities [1, 2]. Species substitution may lead to economic fraud, mislabeling, and violations of religious or ethical dietary requirements [3]. Processed meat products such as sausages are particularly vulnerable to adulteration because morphological characteristics of the original raw materials are lost during processing, making visual identification impossible [4].

Reliable authentication of meat species therefore requires analytical methods capable of detecting species-specific markers in complex and thermally processed food matrices [5]. Various approaches have been proposed for meat authentication, including physicochemical, spectroscopic, immunological, and

molecular genetic techniques [6, 7, 8]. Among these, polymerase chain reaction (PCR)-based methods are considered highly reliable because DNA remains detectable even after substantial technological processing [9, 10].

Mitochondrial DNA markers are particularly suitable for species identification owing to their high copy number and relative resistance to degradation. The cytochrome b (*Cyt b*) gene has been widely used for the authentication of meat products and species differentiation due to the presence of both conserved and species-specific regions. Although several multiplex PCR systems have been described for simultaneous detection of multiple animal species, singleplex PCR assays remain attractive because of their simplicity, reduced risk of primer competition, and ease of result interpretation [11, 12].

The present study was based on the hypothesis that species-specific *Cyt b* primers can provide reliable identification of animal species in thermally processed meat products. Therefore, the objective of this study was to evaluate the applicability and analytical specificity of a *Cyt b*-based PCR assay for detecting declared and undeclared animal species in commercial sausage products.

**Materials and Methods**

Samples and reagents. In this study, muscle tissue from horse meat (n = 5), beef (n = 5), lamb (n = 5), pork (n = 5), and chicken (n = 5) were used as reference samples. In addition, commercial meat products were analyzed, including boiled sausages (n = 4), smoked sausages (n = 7), and frankfurters (n = 4). All samples were stored at −20 °C until DNA extraction.

DNA Extraction and PCR Amplification.

Total DNA was extracted from meat products using the DNA-EXTRAN-2 kit (Syntol, Russia) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 25 µL containing 2 µL of genomic DNA, 1 µL of each primer, 0.5 µL dNTP mixture, 2.5 µL of 10× reaction buffer, 0.2 µL Taq DNA polymerase, and nuclease-free water. Amplification was performed in a T100 thermal cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53–65 °C for 30 s, and extension at 68 °C for 40 s, with a final extension at 68 °C for 5 min. PCR amplification products were purified using the QIAquick PCR Purification Kit (Qiagen, Singapore) according to the manufacturer's protocol. Selected PCR amplicons were subjected to Sanger sequencing using an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). Sequencing data were collected using 3730 DNA Analyzer Data Collection Software v3.0 and analyzed using Sequencing Analysis Software v5.3.1 (Applied Biosystems, USA). Consensus sequences were aligned and compared with reference *Cyt b* sequences retrieved from the GenBank database to confirm species identity.

DNA extracted from reference samples of each animal species served as positive controls. A no-template control containing nuclease-free water instead of DNA was included in each PCR run to monitor possible contamination.

Species-specific primers targeting the *Cyt b* gene, used for PCR amplification, are presented in Table 1.

Table 1. Primers used for amplification of the *Cyt b* gene

| Animal species | Primer sequence (5'→3') | Amplicon size, bp | Annealing temperature (°C) |
|---|---|---|---|
| Horse | F: AGCCACCCTTACCCGATTTTT<br>R: GTCCGCCGATTCATGTTAGTGTC | 500 | 59 |
| Cattle | F: ACGAGGCTTATATTATGGGTCTTA<br>R: ATTGGCCGGGGTGTAGTTATC | 500 | 53 |
| Sheep | F: TCCCAGCCCCCTCAAACAT<br>R: AAAAGCCCCCTCAGATTCATTC | 550 | 60 |
| Pig | F: ACGCAAACGGGGCATCA 3'<br>R: GGGGGAGTGTTAAGTGGGTTTG | 450 | 65 |
| Chicken | F: CCTTTGTGGGCTATGTTCTC<br>R: GGGGGTTACTAGTGGGTTTG | 400 | 55 |

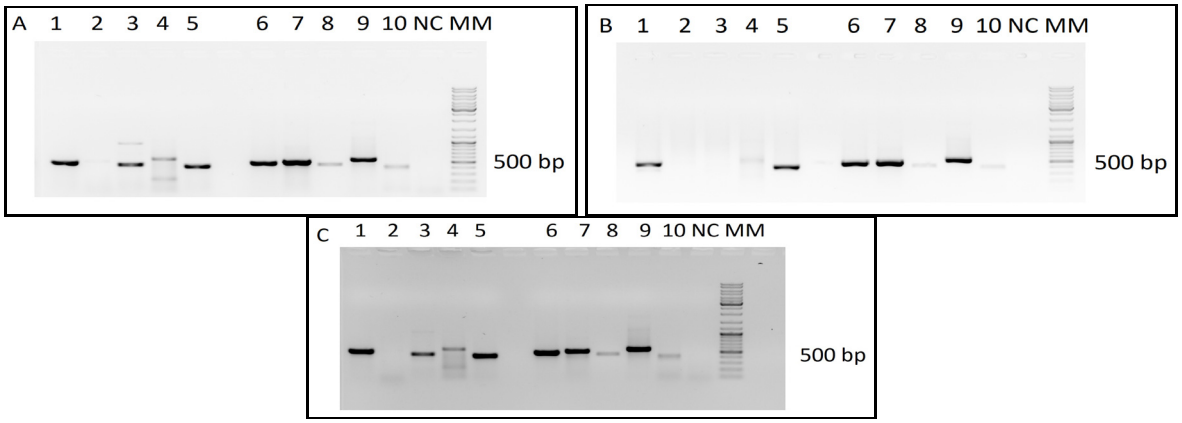
PCR products were analyzed by electrophoresis in a 1% agarose gel. To prepare the gel, 0.4 g of agarose was added to 40 µL of 1× TAE buffer and melted in a microwave oven. The solution was then cooled to 50–60 °C, after which 2.5 µL of ethidium bromide solution was added. The mixture was poured into a casting tray to form a thin gel layer. Using special combs, wells were formed in the gel for sample loading. For electrophoretic analysis, 10 µL of each PCR product was mixed with 2 µL of 6× loading dye and loaded into the agarose gel wells. The electrophoresis was performed under the following conditions: electric field strength of approximately 6 V/cm for 50 minutes. The results of electrophoresis were visualized by UV irradiation of the gel using a transilluminator.

Statistical Analysis

The present study was designed as a qualitative PCR-based species identification assay. Therefore, quantitative statistical comparison of amplification signals was not performed. Results were interpreted based on the presence or absence of species-specific amplicons of the expected size. The proportion of samples showing agreement between declared and detected species composition was calculated descriptively. Because of the limited sample size, estimates of analytical sensitivity, specificity, and limit of detection were not determined and should be evaluated in future studies.

**Results**

PCR analysis of three types of sausage products using species-specific primers targeting the *Cyt b* gene of cattle, horse, pig, sheep, and chicken revealed DNA fragments of 500, 450, and 400 base pairs, as shown in Figure 1. Amplification of the *Cyt b* gene segment in cattle corresponded to a fragment size of 500 base pairs. The amplicon sizes of the mitochondrial DNA fragments of pig and chicken were 450 bp and 400 bp, respectively, which correspond to the expected fragment sizes.



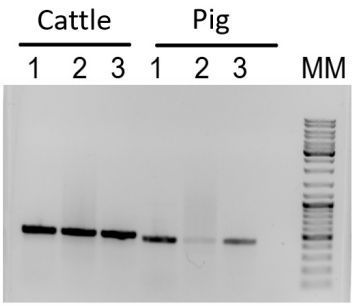

*Note: smoked sausage (A), boiled sausage (B), frankfurters (C)*

Lane 1 – primers targeting *Cyt b* of cattle; Lane 2 – primers targeting *Cyt b* of horse; Lane 3 – primers targeting *Cyt b* of pig; Lane 4 – primers targeting *Cyt b* of sheep; Lane 5 – primers targeting *Cyt b* of chicken; Lane 6 – DNA of cattle; Lane 7 – DNA of horse; Lane 8 – DNA of pig; Lane 9 – DNA of sheep; Lane 10 – DNA of chicken; NC – negative control.

Figure 1. Electrophoregram of PCR analysis of sausage products

Since the composition of the analyzed sausage products did not indicate the presence of pork components, a control PCR analysis was performed using species-specific primers targeting the *Cyt b* gene of cattle and pig.

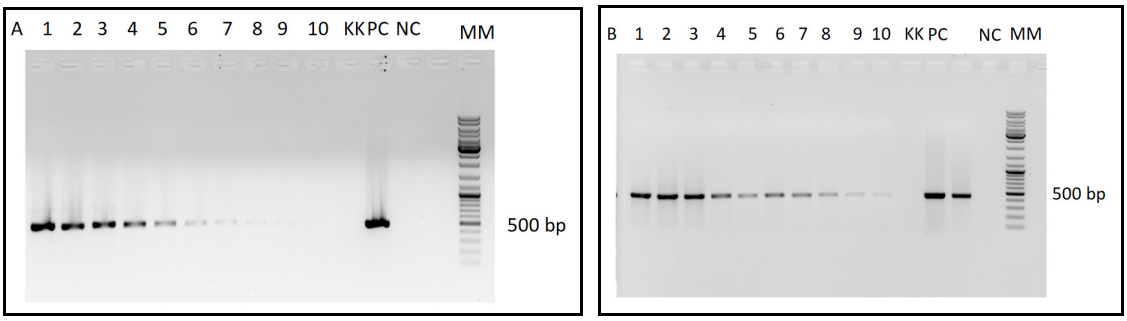
To increase the specificity of the reaction, the annealing temperature was increased to 70 °C, while the optimal annealing temperatures were 53 °C for primers targeting the *Cyt b* gene of cattle and 57 °C for primers targeting the *Cyt b* gene of pig (Figure 2).




Lane 1 – smoked sausage; Lane 2 – boiled sausage; Lane 3 – frankfurters.
Figure 2. Electrophoresis of PCR analysis of sausage products using species-specific primers targeting the *Cyt b* gene of cattle and pig

As shown in Figure 2, the increase in annealing temperature confirms the presence of pig DNA in samples of smoked sausage and frankfurters. In samples of boiled sausage, trace amounts of pig DNA were detected, which may be associated with contamination of production equipment.

The analytical sensitivity of the quantitative PCR assay was assessed using serial dilutions of DNA isolated from animal biological specimens. The assay demonstrated a detection limit ranging from 62.5 pg to 1.9 pg of DNA per 25 µL reaction volume (Figure 3).




Lane 1 – 1000 pg; Lane 2 – 500 pg; Lane 3 – 250 pg; Lane 4 – 125 pg; Lane 5 – 62.5 pg; Lane 6 – 31.2 pg; Lane 7 – 15.6 pg; Lane 8 – 7.5 pg; Lane 9 – 3.9 pg; Lane 10 – 1.9 pg; Lane 11 – 0.9 pg; PC – DNA extracted from beef (positive control); NC – reaction mixture without DNA (negative control); KK – DNA from a non-target animal species; MM – DNA molecular weight marker.
Figure 3. Analytical sensitivity of primers targeting the cytochrome b (*Cyt b*) gene of cattle (A), pig (B)

The simultaneous detection of species-specific amplicons of cattle and pig DNA in the same samples indicates a mixed species composition of the meat raw materials. Differences in band intensity were observed; however, because the assay was qualitative, no conclusions regarding the relative proportion of species-specific DNA were made. The electrophoretic profile indicates the presence of biological material from both cattle and pigs in the sausage products, which is important for quality control, detection of adulteration, and verification of product labeling compliance.

For validation of the PCR analysis based on primers targeting the *Cyt b* gene, four types of boiled sausages, seven types of smoked sausages, and four types of frankfurters were examined. Table 2 presents the results of species identification of meat raw materials in sausage products of various types using PCR based on analysis of the *Cyt b* gene. The declared composition of the products was compared with the actual animal DNA detected in the sausage samples.

Table 2. Results of composition determination in sausage product samples

| No. | Product type | Composition | Animal DNA detected by PCR based on the Cyt b gene |
|---|---|---|---|
| 1 | Boiled sausage No. 1 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Chicken DNA |
| 2 | Boiled sausage No. 2 | Beef<br>Pork meat<br>Spices | Cattle DNA<br>Pig DNA<br>Chicken DNA |
| 3 | Boiled sausage No. 3 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Pig DNA<br>Chicken DNA |
| 4 | Boiled sausage No. 4 | Pork<br>Chicken meat<br>Spices | Pig DNA<br>Chicken DNA |
| 5 | Smoked sausage No. 1 | Pork<br>Chicken meat<br>Spices | Pig DNA<br>Chicken DNA |
| 6 | Smoked sausage No. 2 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Chicken DNA |
| 7 | Smoked sausage No. 3 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Pig DNA<br>Chicken DNA |
| 8 | Smoked sausage No. 4 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Chicken DNA |
| 9 | Smoked (Krakow-style) sausage No. 1 | Chicken meat<br>Spices | Chicken DNA |
| 10 | Smoked (Krakow-style) sausage No. 2 | Pork<br>Beef<br>Spices | Cattle DNA<br>Pig DNA |
| 11 | Smoked (Krakow-style) sausage No. 3 | Beef<br>Spices | Cattle DNA<br>Pig DNA |
| 12 | Frankfurters No. 1 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Chicken DNA |
| 13 | Frankfurters No. 2 | Beef<br>Spices | Cattle DNA |
| 14 | Frankfurters No. 3 | Beef<br>Chicken meat<br>Pork<br>Spices | Cattle DNA<br>Chicken DNA<br>Pig DNA |
| 15 | Frankfurters No. 4 | Beef<br>Chicken meat<br>Spices | Cattle DNA<br>Chicken DNA<br>Pig DNA |

DNA of animal species was successfully detected in all analyzed samples, confirming the suitability of the PCR method based on the *Cyt b* gene for the analysis of thermally processed meat products. The obtained data demonstrate both consistencies and discrepancies between the declared composition and the actual species composition of the sausages. In several samples (boiled sausages No. 1 and No. 4; smoked sausages No. 1, No. 2, and No. 4; frankfurters No. 1–3), the detected DNA corresponded to the meat species indicated on the label. This indicates correct labeling and the absence of substitution of meat raw materials in these products.

At the same time, in samples of boiled sausage No. 2 and No. 3, as well as in smoked sausage No. 3 and smoked (Krakow-style) sausages No. 1–3, the presence of animal DNA not declared in the product composition was detected (pig or chicken DNA in the absence of corresponding labeling). In samples of smoked (Krakow-style) sausage No. 2 and frankfurters No. 4, DNA from several animal species was detected simultaneously, indicating either the use of mixed meat raw materials or possible technological cross-contamination. The detection of chicken DNA in products where it is not declared may indicate a partial substitution of more expensive meat raw materials with cheaper alternatives, or the use of shared production lines without adequate cleaning procedures.

The data presented in the table confirm the high informative value of PCR analysis targeting the *Cyt b* gene for monitoring the authenticity of meat products. The detected discrepancies between the declared and actual species composition indicate possible cases of adulteration or violations of production technology, which highlights the necessity of molecular genetic control within the system of veterinary sanitary inspection and quality monitoring of meat products.

To confirm the presence of pig DNA in sausage products where it was not declared, additional analyses were conducted to isolate and determine the nucleotide sequence of the amplified fragment. Sequencing of selected amplicons showed high similarity to reference *Cyt b* sequences available in GenBank, supporting the species identification results obtained by PCR. (Figure 3).

```
                                                  51                                                  100
NC_012095.1:14164-15303 Sus scrofa domesticus CytB    (49) GCATTCATTGACCTCCCAGCCCCCTCAAACATCTCATCATGATGAAACTT
                                Pig forvard           (48) GCGGGAATTCGATTCCCAGCCCCCTCAAACATCTCATCATGATGAAACTT
                                 Pig revers            (1) ------------TCCCAGCCCCCTCAAACATCTCATCATGATGAAACTT
                                                  101                                                 150
NC_012095.1:14164-15303 Sus scrofa domesticus CytB    (99) CGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAGGCCTGT
                                Pig forvard           (98) CGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAGGCCTGT
                                 Pig revers           (38) CGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAGGCCTGT
                                                  151                                                 200
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (149) TCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTT
                                Pig forvard          (148) TCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTT
                                 Pig revers           (88) TCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTT
                                                  201                                                 250
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (199) ACACACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCTACA
                                Pig forvard          (198) ACACACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCTACA
                                 Pig revers          (138) ACACACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCTACA
                                                  251                                                 300
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (249) TGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCC
                                Pig forvard          (248) TGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCC
                                 Pig revers          (188) TGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCC
                                                  301                                                 350
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (299) GAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGA
                                Pig forvard          (298) GAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGA
                                 Pig revers          (238) GAGGTCTATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGA
                                                  351                                                 400
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (349) GTAGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCT
                                Pig forvard          (348) GTAGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCT
                                 Pig revers          (288) GTAGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCT
                                                  401                                                 450
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (399) GCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACAAATCTAC
                                Pig forvard          (398) GCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACAAATCTAC
                                 Pig revers          (338) GCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACAAATCTAC
                                                  451                                                 500
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (449) TATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGG
                                Pig forvard          (448) TATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGG
                                 Pig revers          (388) TATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGGG
                                                  501                                                 550
NC_012095.1:14164-15303 Sus scrofa domesticus CytB   (499) GGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTTCCACTT
                                Pig forvard          (498) GGCTTTTC----------------------------------------
                                 Pig revers          (438) GGCTTTTAT---CACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATAT
                                                  551                                                 600
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Figure 3. Alignment of the reference nucleotide sequence with the Cyt b DNA fragment isolated from a sausage product

Figure 3 shows that the nucleotide sequences of the *Cyt b* gene fragment isolated from sausage products almost completely coincide with the reference sequence of Sus scrofa domesticus (NC_012095). Throughout the analyzed region, a high degree of nucleotide conservation is observed, which is characteristic of the mitochondrial *Cyt* b gene and confirms its suitability as a marker gene for species identification of meat raw materials. Distinct regions of complementary binding of the forward and reverse primers (Pig forward and Pig reverse) can be clearly identified, indicating the correct primer design and the specificity of PCR amplification. The absence of significant mismatches in the primer-binding regions suggests a high efficiency of amplification of the target pig DNA fragment, even under conditions of technological processing of meat products. Minor nucleotide variations observed at several positions are not systematic and may be attributed to intraspecific variability or sequencing characteristics. Overall, the obtained data demonstrate the high efficiency and specificity of the molecular genetic identification method based on analysis of the *Cyt b* gene.

**Discussion**

The present study demonstrated the applicability of a PCR assay targeting the mitochondrial *Cyt b* gene for qualitative identification of animal species in commercial sausage products. Species-specific amplification products of the expected size were obtained for cattle, horse, sheep, pig, and chicken DNA, indicating successful amplification of the selected target regions. Furthermore, amplification products were detected in thermally processed meat products, confirming the suitability of mitochondrial *Cyt b* markers for analysis of processed food matrices. Analysis of commercial sausage products revealed both agreement and discrepancies between the declared and detected species composition. In several samples, the detected DNA corresponded to the species listed on the product label, whereas in other cases undeclared animal species were identified. The presence of undeclared DNA may indicate substitution of meat raw materials, inaccurate labeling, or technological cross-contamination during processing. However, because the assay was designed as a qualitative method, the relative proportion of each species present in mixed products could not be determined.

To further verify the PCR results, selected amplicons were subjected to nucleotide sequence analysis. Comparison of the obtained sequences with reference *Cyt b* sequences demonstrated high sequence similarity, supporting the species identification results obtained by PCR. These findings are consistent with previous studies reporting the usefulness of mitochondrial *Cyt b* markers for authentication of meat products and detection of species substitution. The practical applicability of the assay was additionally demonstrated through the analysis of commercially available sausage products. The ability to detect animal DNA in thermally processed samples suggests that the method may be useful for routine monitoring of meat authenticity in veterinary and food control laboratories [12].

Several limitations of the present study should be acknowledged. First, the assay was evaluated as a qualitative method and does not provide quantitative information regarding the proportion of meat species in mixed products. Second, cross-reactivity was evaluated only for the investigated animal species and was not assessed using DNA from additional species. Third, technical PCR replicates were not performed, and therefore assay reproducibility was not formally evaluated.

In addition, the relatively small number of reference samples does not allow estimation of diagnostic sensitivity and specificity with statistical confidence. Blinded evaluation of samples was not performed, as the declared composition of commercial products was known to the investigators during analysis. Although species identification was based on objective PCR amplification and sequence analysis results, the possibility of observational bias cannot be completely excluded. Future studies should therefore focus on determination of the limit of detection, assessment of assay reproducibility, blinded sample analysis, evaluation of analytical performance characteristics, and validation using larger sample sets and experimentally prepared meat mixtures.

**Conclusion**

The present study demonstrated the applicability of a *Cyt b*-based PCR assay for qualitative identification of cattle, horse, sheep, pig, and chicken DNA in commercial sausage products. The method successfully detected both declared and undeclared animal species in thermally processed products. The obtained results indicate that *Cyt b*-based PCR may serve as a useful tool for routine meat authenticity testing. However, further studies are required to determine analytical sensitivity, limit of detection, and diagnostic performance characteristics of the assay.

**Authors' Contributions**

SB, AB: Planned the study and drafted and revised the manuscript. KT, AS: Performed the extraction of DNA and PCR analysis. KM, AB: Performed the analysis date. All authors have read, reviewed, and approved the final manuscript.

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During the preparation of this manuscript, the authors used ChatGPT (OpenAI) for language editing and improvement of grammar. The authors reviewed and edited the output and take full responsibility for the content of the publication.

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**Conflicts of Interest**

The authors declare that they have no conflicts of interest. Aitbay Bulashev serves as the Scientific Editor of CAJVS; however, he did not participate in the peer review process or in the decision-making regarding this manuscript.

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