The Seybold Report

ISSN 1533-9211

DOI 10.17605/OSF.IO/D6YBV

MULTIPLEX PCR ASSAY FOR ADULTERATION CHECK IN LOCAL CHEESE PRODUCTS

ISSA MOUSA MOHAMMED

Department of Food Science and Technology, College of Agricultural Engineering Sciences, University of Duhok, Iraq. Corresponding Author Email: issa.mousa@uod.ac

Abstract

Food fraud, especially milk, has been the main problem in most areas of the world. Techniques have been developed to avoid such adulteration. One of these techniques is PCR which is considered fast, accurate, and reliable. In this study, 42 local white cheese samples were collected from different farmers and dairy markets in Duhok province, north of Iraq. A multiplex PCR assay was applied to examine these cheese samples. Three sets of primer were used to amplify specific 12s and 16s rRNA of the sheep, goat, and cow DNA through multiplex PCR reaction. In the results, 95.23% of the collected samples were adulterated. Only 4.67% of the samples were according to the labeling provided by the farmers and/or dairy markets. The most used milk for cheese production was goat followed by cow and sheep milk with 51.56%, 37.5%, and 10.93%, respectively. None of the 40 samples which were declared to be pure sheep cheese were free of adulteration. These data conclude that cheese fraud is high in this region and needs a possible solution in terms of maintaining such regulations and laws to protect consumers from adulteration.

Keywords: Cheese; DNA; Adulteration; Multiplex-PCR; Iraq

INTRODUCTION

The "Farm to Fork" principle relies on the authenticity and traceability of a product from its source to the consumer (Santos et al., 2003). The agri-food sectors and government agencies in charge of assuring the quality and safety of food have increasingly focused on preventing food fraud (Baptista et al., 2021). Milk and dairy products are currently the most commonly adulterated food items, mostly because of the nutritional significance of milk, worldwide consumption, shortened shelf life, and lack of innovative techniques for product authentication (Stadler et al., 2016). Even though the establishment of strict food standards (such as British Retail Consortium (BRC) Food and International Organization for Standardization (ISO) standards) has made it increasingly easier to ensure the security and reliability of food products in supply chains, food fraud cases have increased due to globalization and the expansion of these chains (Banati, 2014). The majority of milk food fraud instances include the unintentional replacement of a high-priced good (such as sheep, buffalo, or goat's milk) with a cheaper or lower-quality substitute (such as cow milk, or milk from less valued breeds), or the removal of a claimed milk species (Baptista et al., 2021). Other reasons for milk substitution are personal preference (Hurley et al., 2004); religion, or cultural issues (Shatenstein and Ghadirian, 1998).

To identify the species of milk and dairy products, a variety of analytical techniques have been used. Among these, are immunological (Xue et al., 2010), electrophoretic (Mayer, 2005), and chromatographic (Enne et al., 2005). Recently, the focus has shifted to DNA-based techniques for a variety of food authentication applications, including the detection of milk product adulteration (Woolfe and Primrose, 2004). In terms of time, cost, sample volume, and sample

The Seybold Report
ISSN 1533-9211
DOI 10.17605/OSF.IO/D6YBV

processing, DNA-based approaches outperform protein-based methods. For the identification of species in raw meat, meat products, fish, and dairy products, Polymerase Chain Reaction (PCR) is one of the most used molecular biology methods (Veloso et al., 2002). Even processed cheese and milk still contain genomic DNA from somatic cells. Targeting DNA sequences with sufficient species-species variance, PCR assay of DNA isolated from somatic cells in milk has been effectively employed to identify adulteration of milk products (Maudet and Taberlet, 2001; Rea et al., 2001; Bottero et al., 2003). Since the sequences of even closely related species might vary by several nucleotides, many of these investigations use mitochondrial-encoded genes, such as the cytochrome b gene (Bottero et al., 2003). None of these techniques is used locally for species identification. Therefore, this study aims to use a PCR-based assay for species identification or adulteration measurement in locally-made cheese and its products.

MATERIALS AND METHODS

Sample collection

For the sample collection, 42 fresh cheese samples were collected from local supermarkets and directly from farmers in Duhok province, north of Iraq. The samples were unlabelled; however, the labeling process depended on the owner's trust. Out of these samples, 40 were supposed to be from sheep species, one sample from cow, and one sample from goat and sheep. The samples were transported directly in cold conditions to the animal production laboratories, college of agricultural engineering sciences, University of Duhok.

DNA extraction

Total nucleic acids were extracted from cheese with the AddPrep Genomic DNA Extraction Kit (add bio, Daejeon, Republic of Korea) with some modifications. 100 mg of cheese was ground in 500 ml of PBS and the mixture was centrifuged at 10,000 rpm for 10 seconds. 200 µl of the middle phase was taken for DNA extraction. Next, all the steps were performed according to the guidelines of the manufactured kit. The elution was done with 60 µl of elution buffer instead 0f 100 µl. The quality and quantity of extracted DNA samples were measured with a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc, and USA). They were stored at -20 ºC.

Primers and multiplex PCR reaction

For the species detection of cow, sheep, and goat in cheese three pairs of species-specific primer targeting 12s and 16s of mitochondrial rRNA gene was used (Table 1). These primers were designed and validated by Buttero et al. (2003). A final volume of 50 µl reaction was made containing 1X AddStart Taq Master Mix, 15 pmol/µl of each primer, and 50-250 ng/µl of DNA. The reaction program was set in GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Singapore) as follows; after an initial denaturation step at 94 oC for 5 minutes, 35 cycles were performed as follows: denaturation at 94 oC for 30 seconds, annealing at 55 ºC for 60 seconds, extension at 72 oC for 60 seconds, one final extension cycle at 72 oC for 5 minutes. The amplicons were resolved in 2.5% agarose electrophoresis at 100 volts for 30 minutes.

The Seybold Report
ISSN 1533-9211
DOI 10.17605/OSF.IO/D6YBV

Table 1: The primer sequences used in multiplex PCR reaction

Primer name	Sequence	Amplicon size (bp)
Sheep-F Sheep-R	5-ATATCAACCACACGAGAGGAGAC-3 5-TAAACTGGAGAGTGGGAGAT-3	172
Goat-F Goat-R	5-CGCCCTCCAAATCAATAAG-3 5-AGTGTATCAGCTGCAGTAGGGTT-3	326
Cow-F Cow-R	5-GTACTACTAGCAACAGCTTA-3 5-GCTTGATTCTCTTGGTGTAGAG-3	256

RESULTS

The modified protocol of DNA extraction from cheese was somehow efficient in providing enough DNA for downstream application. The genomic DNA in agarose gel electrophoresis was highly faint and was not possible to show the data. Although the NanoDrop measurements for the genomic DNA were poor, especially the 260/230 parameters and concentrations, the PCR was able to obtain and amplify the targeted amplicons for all three selected species. The specific amplicons 172 bp, 256 bp, and 326 bp were amplified for sheep, cows, and goats, respectively (Figure 1). Out of 42 collected cheese samples only two (4.76%) of them were according to the declared species and the others 40 (95.23%) were fraud and contained secondary species. Of the fraud samples (40 samples), 3 (7.5%) were a mixture of sheep and goat sources. 14 (35%) were from goat sources only. 8 (20%) were from cow sources only. 12 (30%) were a mixture of cow and goat sources. 3 (7.5%) were a mixture of three species (cow, goat, and sheep). The usage of goat milk in cheese manufacturing was highest among the collected samples at 51.56% followed by cow milk at 37.5% and finally, the lowest milk source for cheese production was sheep source with 10.93%.

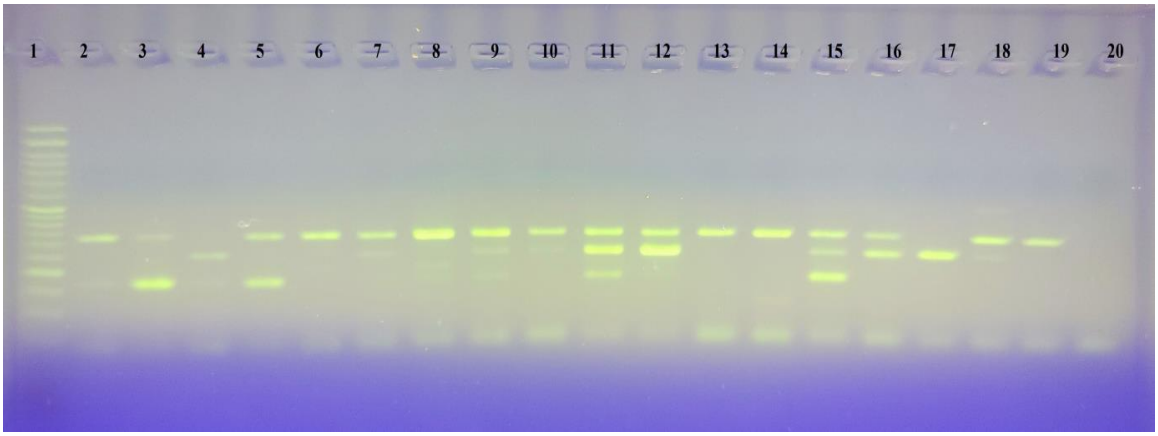

Figure 1: Agarose gel electrophoresis for multiplex PCR reaction amplifying 172 bp, 256 bp, and 326 bp of sheep, cow, and goat species, respectively. Lane 1: 50bp DNA ladder, Lane 2-19: cheese samples, and Lane 20: Negative control

The Seybold Report
ISSN 1533-9211
DOI 10.17605/OSF.IO/D6YBV

DISCUSSION

Food fraud is becoming a major issue on a global scale, mostly due to the food industry's fast innovation and the shifting preferences of consumers. As a result, it became more important to develop and implement trustworthy authentication procedures, which led to the replacement of protein-based dairy product verification techniques with more accurate and repeatable DNA-based approaches Baptista et al., (2021). Therefore, it's critical to safeguard the interests of the customer by putting in place proper control mechanisms and equipping food analysts with the tools they need to identify adulterated milk. There may be regional variations in labeling and authenticity laws, necessitating the use of analytical testing to enforce such laws (Dennis, 1998). Besides several researches on microbial and physicochemical properties of marketed milk and cheese products (Ali et al., 2020; Nanakali et al., 2023; Mustafa 2023), there is no available data on local cheese adulteration check in this region. However, many studies have been performed in other countries. Khanzadi et al., (2013) found 80% of the tested dairy samples in Mashhad City, Iran were fraudulent and only 20% were containing pure sheep milk. Similarly, Tsirigoti et al., (2020) examined several dairy products in Greece and they found that 37.5% of cheese and 45% of yogurt samples were containing undeclared milk. Colak et al., (2006) and Zelenakova et al. (2009) found 48% and 40% of sheep cheese was mixed with cow milk, respectively.

Several methods are developed for this purpose; for example the protein-based techniques. However, these techniques have many disadvantages such as cheese maturation and heat treatment may denature the protein (Plath et al., 1997). Additionally, the protein assays for species identification are laborious and time-consuming (Karoui and Baerdemaeker, 2007).

Recently, full attention has been put on more reliable and fast assays based on DNA for species identification in food. One of these assays is Polymerase Chain Reaction (PCR) which depends on nucleic acid in the somatic cells that persist in the cheese even after heat and manufacturing of the cheese (Diaz et al., 2007). Among the targeted genes, mitochondrial genomic DNA, especially 12s and 16s rRNA, is the most widely used for this purpose (Bottero et al., 2003). However, there are some challenges to DNA extraction from cheese. Because the somatic cells are low in the milk, therefore low DNA concentration may be expected in the cheese. In addition, the high fat and protein percentage in the cheese causes a problem and may lower the purity of the extracted DNA (Baptista et al., 2021). In the present study, the DNA extraction procedure was modified to collect as many somatic cells as possible, while the purity and concentration of the DNA were below the optimum parameters for the downstream application. Although the primers were designed by Bottero et al., (2003) for the European breeds, they were used in this study and were successful in amplifying the specific targets of the DNA in the PCR for the Iraqi breeds. In the studied region, north of Iraq, labeling of the local white cheese is not compulsory and consumers depend mostly on the farmer’s trust. Based on this, samples were collected and most of them were identified to be pure sheep-made cheese. Compared to similar research on cheese adulteration checks, our results showed the highest miss-labeling data in which more than 95% of the studied cheese samples were containing secondary milk sources.

The Seybold Report
ISSN 1533-9211
DOI 10.17605/OSF.IO/D6YBV

CONCLUSION

In conclusion, the used assay for species identification is fast, and reliable in terms of sensitivity and accuracy. The adulteration of the studied cheese samples was over the expected range. None of the declared pure sheep cheese samples were free of second or third milk sources. Laws and regulations must be put to protect the consumer's rights and avoid such fraud in the markets.

Declaration of Interest

The author declares no competing interest.

References

1. Banati, D. (2014). European perspectives of food safety. Journal of the Science of Food and Agriculture, 94(10), 1941-1946.
2. Baptista, M., Cunha, J. T., & Domingues, L. (2021). DNA-based approaches for dairy products authentication: A review and perspectives. Trends in Food Science & Technology, 109, 386-397.
3. Bottero, M. T., Civera, T., Nucera, D., Rosati, S., Sacchi, P., & Turi, R. M. (2003). A multiplex polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. International Dairy Journal, 13(4), 277-282.
4. Colak, H., Aydin, A., Nazli, B., & Ergun, O. (2006). Detection of presence of cow's milk in sheep's cheeses by immunochromatography. Food Control, 17(11), 905-908.
5. Dennis, M.J (1998). Recent developments in food authentication. Analyst, 123(9), 151R-156R.
6. Díaz, I. L. C., Alonso, I. G., Fajardo, V., Martín, I., Hernández, P., Lacarra, T. G., & de Santos, R. M. (2007). Application of a polymerase chain reaction to detect adulteration of ovine cheeses with caprine milk. European Food Research and Technology, 225, 345-349.
7. Enne, G., Elez, D., Fondrini, F., Bonizzi, I., Feligini, M., Aleandri, R. (2005) High-performance liquid chromatography of governing liquid to detect illegal bovine milk addition in water buffalo Mozzarella: Comparison with results from raw milk and cheese matrix. J Chromatogr. A1094: 169-178.
8. Karoui, R., & De Baerdemaeker, J. (2007). A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products. Food chemistry, 102(3), 621-640.
9. Khanzadi, S., Jamshidi, A., Razmyar, J., & Mohsenzadeh, M. (2013). PCR-based detection of cow and goat milk in sheep milk and dairy products marketed in Mashhad city of Iran. Iranian Journal of Veterinary Medicine. 7(4):257-262
10. Mafra, I., Ferreira, I. M., Faria, M. A., & Oliveira, B. P. (2004). A novel approach to the quantification of bovine milk in ovine cheeses using a duplex polymerase chain reaction method. Journal of agricultural and food chemistry, 52(16), 4943-4947.
11. Maudet, C., & Taberlet, P. (2001). Detection of cows' milk in goats' cheeses inferred from mitochondrial DNA polymorphism. Journal of Dairy Research, 68(2), 229-235.
12. Mayer, H.K. (2005) Milk species identification in cheese varieties using electrophoretic, chromatographic and PCR techniques. Int Dairy J. 15: 595- 604.
13. Plath, A., Krause, I., & Einspanier, R. (1997). Species identification in dairy products by three different DNA-based techniques. Zeitschrift für Lebensmitteluntersuchung und-Forschung A, 205, 437-441.
14. Rea, S., Chikuni, K., Branciari, R., Sangamayya, R. S., Ranucci, D., & Avellini, P. (2001). Use of duplex

The Seybold Report
ISSN 1533-9211
DOI 10.17605/OSF.IO/D6YBV

polymerase chain reaction (duplex-PCR) technique to identify bovine and water buffalo milk used in making mozzarella cheese. Journal of Dairy Research, 68(4), 689-698.

15. Santos, J., Fernandes, P., & Bardsley, R. (2003). Portuguese "PDO "cheese and species origin of milk. Electronic Journal of Environmental, Agricultural and Food Chemistry, 2, 476-479.
16. Shatenstein, B., Ghadirian, P. (1998) Influences on diet, health behaviors and their outcome in select ethnocultural and religious groups. Nutrition. 14: 223-230.
17. Stadler, R. H., Tran, L. A., Cavin, C., Zbinden, P., & Konings, E. J. M. (2016). Analytical approaches to verify food integrity: Needs and challenges. Journal of AOAC International, 99(5), 1135–1144.
18. Tsirigoti, E., Katsirma, Z., Papadopoulos, A. I., Samouris, G., Ekateriniadou, L. V., & Boukouvala, E. (2020). Application of triplex-PCR with an innovative combination of 3 pairs of primers for the detection of milk's animal origin in cheese and yoghurt. Journal of Dairy Research, 87(2), 239-242.
19. Veloso, A. C., Teixeira, N., & Ferreira, I. M. (2002). Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea–polyacrylamide gel electrophoresis: Detection of milk adulterations. Journal of Chromatography A, 967(2), 209-218.
20. Woolfe, M., Primrose, S. (2004) Food forensics: using DNA technology to combat misdescription and fraud. Trends Biotechnol. 22: 222-226.
21. Xue, H., Hu, W., Son, H., Han, Y., Yang, Z. (2010) Indirect ELISA for detection and quantification of bovine milk in goat milk. J Food Sci Technol. 31: 370-373.
22. Zelenakova, L., Sestak, M., Zidek, R. (2009) Monitoring of sheep milk and milk products adulteration on Common European food market. Potravinarstvo. 3: 69-73.