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Production of Multiplex PCR kit for fraud detection in dairy products

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Abstract

In recent years, along with the growing purchasing power of the population, there has been an increasing demand for high quality product. The object of this study was the identification fraud and animal species (cattle, ovine and caprine) in the dairy products by using Multiplex PCR method. 30 different samples (milk, yogurt and cheeses) were collected from companies and local product. DNA extraction was done based on the guanidinium thiocianate-silicagel method. PCR tests were specific for each species and allowed detection in feedstuffs of levels as low as 1% cattle, ovine and caprine DNA. The length of the amplified fragments with bovine primer is 470 bp, ovine 584 bp and caprine 330 bp which are equal to the expected range. The results showed that 100% of the ovine and caprine cheeses were adulterated with bovine's milk. 50% of ovine milk samples and 60% of ovine yoghurt samples were contaminated with bovine's milk. Also, the high sensitivity of Multiplex PCR in identification of animal species and detection of dairy products fraud was shown.

Key words : Adulteration, Detection, Multiplex PCR

INTRODUCTION

People's tendency to consume healthy and functional foods has caused a higher level of public health and has also improved and expanded the food industry [1]. Due to an ever increasing population and along with it, the need for more animal products, a large number of food manufacturers have always tried to find ways to reduce the cost of raw materials, so in this way they can subsequently increase their financial interests [2]. These solutions are not always reasonable and there are profiteers who reduce the product quality through fraud. Besides, nutrition facts labels will not adequately and properly ensure the actual composition of food products. Therefore there need to be methods to identify and authenticate the food composition so that all consumers and producers are protected against illegal ersatz substitution and adulteration [3]. Detection of such contamination in world has recently received considerable attention. In order to achieve deterrent laws, there need to be control and analysis tools so that legislators would be able to monitor strict enforcement and recognizes fraud and cheating [4].

The consumption of meat and dairy products and their role in providing public health has gained considerable attention in recent years [5, 6]. Currently, different dairy products in the world have different prices. In Iran, for example, dairy products manufactured from ovine are more desirable than those from bovine in terms of taste and therefore forgers mix ovine's milk with some caprine or bovine's milk and then sell it.

Significant advances in biotechnology in recent years and high precision of PCR-based molecular techniques have developed evaluation methods that are fast and precise enough to detect fraud and identify a variety of animal species (bovines, ovine and caprines) used in human dietary protein products such as meat and dairy products [7-9]. Due to its advantages, mitochondrial DNA is preferable to genomic DNA for species recognition and detection of fraud in industrial processed products and livestock and poultry feeds [10].

MATERIAL AND METHODS

Bioinformatics studies

To find genes related to bovine's, ovine's and caprine's mitochondria, the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) was sought and then whole mitochondrial genomes of three species *Bos primigenius*, *Ovis aries* and *Capra hircus* were obtained. CLC Work Benche 5.5 software was used for gene analysis. Also, the website (http://www.sacs.ucsf.edu/cgi-bin/multalin.py) was used for multi-alignment. After multi-alignment of whole mitochondrial genomes of the three species, the cytochrome b region was considered suitable for primer design.

Preparation and collection of the samples

For Positive control, 5 ml of blood was collected from each of the bovines, ovine and caprines in the Research Station, Ferdowsi University of Mashhad. Three types of dairy products (milk, yogurt and cheese) in 30 samples were purchased from various shops in Mashhad and Quchan, Iran, provided that different manufacturers had produced them. All samples were stored in a freezer at -20 °C until DNA extraction.

DNA extraction

DNA was extracted by Gene JET PCR Purification Kit # K0721 was designed by Thermo with modifications. 200 mg of the cheese and yogurt samples were transferred to 1.5 mL microtubes. Then 400 microliters of lysis buffer with 40 microliters of proteinase K were added to the mixture and was stirred using a vortex. Microtubes were incubated for 16 hours in Benmary (37 ° C). Samples were centrifuged for 30 min at 10000 rpm. After centrifugation, a three-phase solution was resulted the middle phase of which was separated for further extraction. For milk sample, 15 ml of the milk sample was discharged. Samples were centrifuged for 30 min at 10000 rpm. After centrifugation, a three-phase solution was obtained and the middle phase was separated for further extraction steps. After this step, in order for the DNA to precipitate, 100 ml isopropanol was added to the

microtubes and stirrerd. The product obtained was transferred to a tank at the top of binding columns. Following this stage, the microtubes were centrifuged for one minute at 8000 rpm. After centrifugation, the column was transferred to new 2 mL microtubes. 500 microliters of W1 buffer was added to the mixture and again centrifuged for one minute at 8000 rpm. After this stage, 500 microliters W2 was added to the mixture and centrifuged for one minute at 8000 rpm. Microtubes were once again centrifuged at 12000 rpm for one minute for complete removal of ethanol. The binding column was transferred to new 1.5 ml microtubes. 100 ml elution buffer (EL) was added to the binding column. After 5 minutes, the microtubes were centrifuged at 8000 rpm for a minute to separate the DNA from the columns. The product was obtained in about 100 microliters.

Multiplex PCR

Species-specific primers for sequence amplification of mitochondrial Cyt b genome were designed using the software Primer Premier 5. Four specific primers were designed according to the sequence homology of mitochondrial Cyt b genes in bovines, ovine and caprines using the software CLC Workbench 5.5. Table 1 shows the features of designed forward and reverse primers for amplification of mitochondrial Cyt b genome. The primers used in this study were purchased from MWG, Inc. (Germany), and diluted with TE buffer for preparing a stock solution, and stored at - 20°C before used. Two microliters of template DNA were added to 23 µl of the PCR mixture, containing Amplification buffer 10X 2.5µl, dNTP mixture (20mM) 2 µl, MgCl2 (50 mM) 1.3 µl, Multiplex primer (20 µM of each) 2µl and 2 U of Taq DNA polymerase. The PCR was performed in a thermo cycler (Biometra, Germany) under the following conditions: an initial denaturation step at 94°C for 30 sec, annealing at 62°C for 35 sec and extension at 72°C for 40 sec, for 35 cycles, followed by a final extension time at 72°C for 10 min. PCR and negative control extractions were included for each amplification experiment, in order to detect false positives due to contamination. After the reaction, samples were electrophoresed on 2% agarose gel.

RESULTS

Bioinformatics results

After reviewing the results of three species multi-aligns, bovine, ovine and caprine, the Cytb gene of these species was selected in order to produce PCR multiplex kit. Although 12sRNA and 16sRNA genes were completely protected and used in some research, the Cytb gene was opted as a result of having more appropriate sites for the primer to anneal to. The Nucleotide sequence and the primers binding sites can be seen in figure 1. After sequencing, it was shown that the Sse9I enzyme is capable of cutting each of the three sequences and it is possible to use RFLP PCR to optimize detection conditions. As it can be seen in figure 1, it is expected that the PCR product for bovine, ovine and caprine species would be 470, 584 and 330 bp on agarose gel respectively. With the purpose of producing Multiplex PCR kit, the reverse primer (Universal) was designed similarly for the three species.

DNA extraction quality

Electrophoresis of products of blood, milk, yoghurt and cheese DNA extraction

The DNA extracted produced large molecules with approximately 15-16 kilobase and indicated that the size of these molecules are suitable for molecular research. Clarity and intensity of bands represents high DNA concentration. Figure 2 shows blood extracted DNA bands after electrophoresis on 0.8 % agarose. Figure 3 shows milk and yoghurt extracted DNA bands after electrophoresis on 0.8 % agarose gel. Figure 4 shows cheese extracted DNA bands after electrophoresis on 0.8 % agarose gel.

Multiplex PCR results

Results of PCR products electrophoresed on 2% agarose gel are shown in figure 5 and figure 6. As can be seen in figures 5, the length of the amplified fragments with bovine primer is 470 bp, ovine 584 bp and caprines 330 bp which are equal to the expected range. This indicates that firstly the primers were designed and selected suitably and secondly, PCR was performed correctly and precisely. It would also suggest that the selected primers only had one binding site on target DNA and there is no sequence identity elsewhere, because a non-specific band, Smear or dimer was not observed and the bands were all clear. Negative control indicates that PCR was performed without contamination. In this study it was found that Multiplex PCR can replace simple PCR due to its accuracy and speed to test suspicious samples and provide a definitive answer. Results from dairy products using a Multiplex PCR are summarized in Table 3. The results showed that 100% of the ovine and caprine cheeses were adulterated with bovine's milk. 50% of ovine milk samples and 60% of ovine yoghurt samples were contaminated with bovine's milk.

Discussion

Murphy et. al. [11] reported that no DNA extraction method for dairy products can provide DNA in large scale for specialists. They believed that adding EDTA to milk causes calcium to chelate, which results in digestion of casein. However, today using powerful commercial kits, DNA extracts possess high quality and are appropriate for quick PCR assays. The method suggested in this study can only be used when extraction is done manually. In some studies, researchers used phenol chloroform

Table 1: Features of forward and reverse primers.

Gene	Primer	Primer length	Product
Bovine's forward primer	5' TCCTTCCATTTATCATCATAGCAA 3'	24	472
Ovine's forward primer	5' TACCAACCTCCTTTCAGCAATT 3'	22	585
Caprine's forward primer	5' CGCCATGCTACTAATTCTTGTT 3'	22	330
Universal Reverse primer	5' TCCTTCCATTTATCATCATAGCAA 3'	23	-

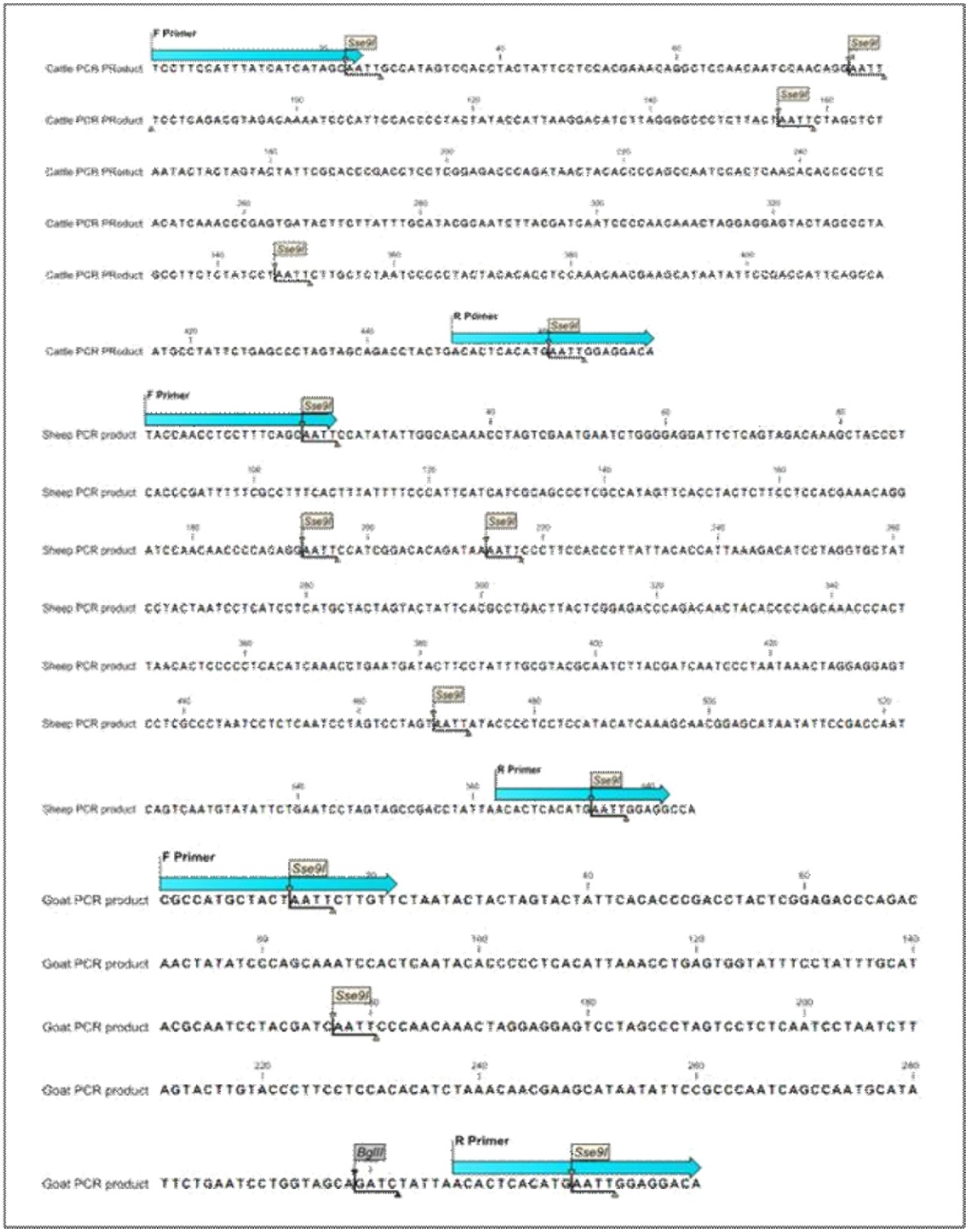

Fig. 1: The expected PCR product of cytochrome b gene of three species, bovine, ovine and caprine.

isoamyl alcohol method and some others used commercial DNA extraction kits [10, 12-13]. DNA extracted using chloroform method does not work on PCR very well, since it has some pollution. In another study, Dalmasso *et al*. and Bottero *et al*. [10, 14] utilized Multiplex PCR assay for detection and identification of species used in food, dairy, and MBM products. They were able to prove the existence of fraud in some samples of food. Also, Di Pinto's [15] studied adulteration in the sausages produced in Italy based on PCR-based techniques. The labels indicated the sausage was made from horse meat. They stated that of the 30 sausage samples that were tested 6 samples were a mixture of horse meat and pork and in one instance there was no horse meat. Zarringhaba *et al*. [16] studied detection of adulteration in meat using Multiplex PCR. They described this method as simple, rapid and inexpensive.

CONCLUSION

PCR has been presented as a sensitive, simple and inexpensive method to detect adulteration in food products based

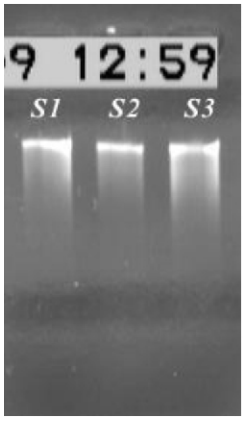

Fig. 2: Quality assessment of blood DNA extract using electrophoresis on 0.8 % agarose.S1, S2 and S3 samples belong to blood DNA extracts of bovine, ovine and caprine, respectively.

Fig. 3: Quality assessment of milk and yoghurt DNA extract using electrophoresis on 0.8 % agarose.S1 to S4 samples belong to yoghurt and S5 to S8 belong to milk.

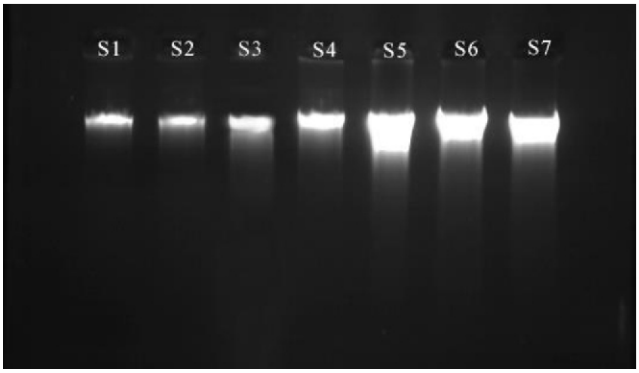

Fig. 4: Quality assessment of cheese DNA extract using electrophoresis on 0.8 % agarose.S1 to S7 samples belong to Ghuchan ovine, Guchan caprine, Guchan bovine, Mashhad Kurdish, Mashhad Lighavan, Tabriz Lighavan, Mashhad Laktiki cheese.

on animal species. With this aim, production of a fraud detection kit for meat and dairy products was tested. Regarding the applicability of the results found to presuppositions in this study, manipulation of Simplex and Multiplex PCR is a very powerful tool for investigating fraud detection in meat and dairy products using mitochondrial DNA sequence and in general genetic studies and detection of different animal species. It is worth mentioning that the method presented in this paper can easily present a commercial kit for detection of adulteration in food. Although the main material tested in this study has been dairy products, our

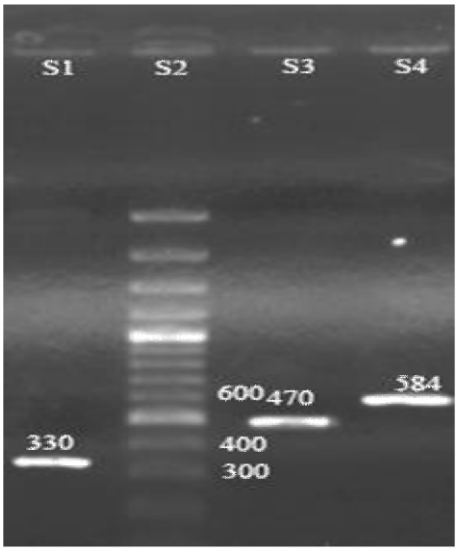

Fig. 5: Specificity of the PCR assay for detection of caprine, bovine and ovine DNA using electrophoresis on 0.8 % agarose. S1: Caprine sample, S2: M100 Marker, S3: Bovine sample and S4: Ovine Sample.

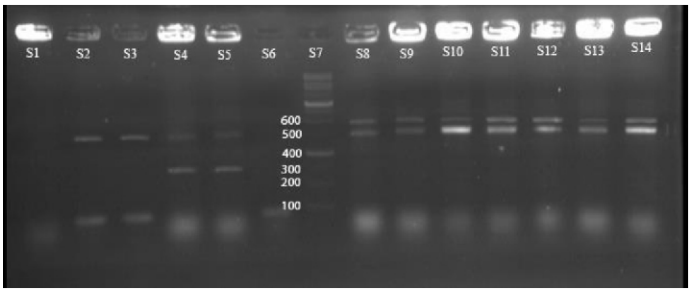

Fig. 6: Detection of the inclusions of cow milk in goat and ovine cheese by Multiplex PCR. S1, S6: negative control, S2, S3: cow milk, S4, S5: Caprine cheese and yogurt samples, S7: M100 Marker, S8-S14: Ovine cheese and yogurt samples.

Table 2: Occurrence of target primers in different samples

Food sample	number of samples	number of positive samples with bovine primer	number of positive samples with ovine primer	number of positive samples with caprine primer
Cheese	9	9	3	1
Yoghurt	12	10	3	1
Milk	9	7	2	2

results suggest that this kit can easily detect protein products, meat and even animal products as well.

REFERENCES

1. Bellagamba F., V. M. Moretti, S. Comincini, F. Valfre. Identification of species in animal feedstuffs by polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial DNA. Agric Food Chem. 2001: 49: 3775-81.

2. Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci, P. A. Battaglia. Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. Food Prot. 1998: 61: 513-8.

3. Myers, M. J., D. E. Farrell, D. N. Heller, and H. F.Yancy. Development of a polymerase chain reaction-based method to identify species-specific components in dog food. Am J Vet Res. 2004: 65: 99-103.

4. Kyle, C. J., C. C. Wilson. Mitochondrial DNA identification of game and harvested freshwater fish species. Forensic Sci. Int. 2007: 166(1): 68-76.

5. Burger, J., R. Schoon, B. Zeike, S. Hummel, B. Herrmann. Species determination using species-discriminating PCR-RFLP of ancient DNA from prehistoric skeletal remains. Ancient Biomolecules. 2002: 4: 19-23.

6. Pfeiffer, I., J. Burger, B. Brenig. Diagnostic polymorphisms in the mitochondrial cytochrome b gene allow discrimination between cattle, sheep, goat, roe buck and deer by PCR-RFLP. BMC Genetics. 2004: 5: 30.

7. Matsunga, T., K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada, Y. Shinmura. A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Sci. 1999: 51: 143-148.

8. Wolf, C., J. Luthy. Quantitative competitive (QC) PCR for quantification of porcine DNA. Meat Sci. 2001: 57: 161-168.

9. Rea S, Chikuni K, Branciari R, Sangamayya RS, Ranucci D, Avellini P. Use of duplex polymerase chain reaction (duplex-PCR) technique to identify bovine and water buffalo milk used in making mozzarella cheese. Dairy. Res. 2001: 68(4): 689-98. PubMed PMID: 11928964.

10. Dalmasso, A., E. Fontanella, P. Piatti, T. Civera, S. Rosati, M. Bottero. A multiplex PCR assay for the identification of animal species in feedstuffs. Mol. Cell. Probes. 2004: 18: 81-87.

11. Murphy A. M, M Shariflou, C. Moran. High Quality Genomic DNA extraction from large milk samples. Dairy Research. 2002: 69: 645-649.

12. Bottero, M. T., T. Civera, A. Anastasio, R. M. Turi, S. Rosati. Identification of cow's milk in "buffalo" cheese by duplex polymerase chain reaction. Food Protection. 2002: 65: 362366.

13. Che Man, Y. B., A. A. Aida, A. R. Raha, R. Son. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. Food Control. 2007: 18(7):885-889.

14. Bottero, M. T., A. Dalmasso, D. Nucera, R. M. Turi, S. Rosati, S. Squadronne, M. Goria, T. Civera. 2003. Development of a PCR assay for the detection of animal tissues in ruminant feeds. Food Protection. 2003: 66: 2307-2312.

15. Di Pinto, A., V. T. Forte, M. C. Conversano, G. M. Tantillo. Duplex polymerase chain reaction for detection of meat in horse meat fresh sausages from Italian retail sources. Food Control. 2005: 16: 391-394.

16. Zarringhabaie GE., N. Pirany, A. Javanmard. Molecular traceability of the species origin of meats using multiplex PCR. African Journal of Biotechnology. 2011: 73: 15461-16465.