Asian-Aust. J. Anim. Sci.
Vol. 19, No. 10 : 1503 - 1507
October 2006

www.ajas.info

Investigation of Goats' Milk Adulteration with Cows' Milk by PCR

Yeong-Hsiang Cheng[1], Su-Der Chen[2] and Ching-Feng Weng*

[1]College of Bioresources, Department of Animal Science, National I-Lan University, I-Lan, Taiwan, ROC

ABSTRACT : Goats' milk adulteration with cows' milk is becoming a big problem. In the past, the urea-polyacrylamide gel electrophoresis assay with different motility of αS1-casein has been applied for the identification of cows' milk adulteration. The detection sensitivity is 1.0%. The aim of this study was to develop a faster and more sensitive method to detect cows' milk which may be present in adulterated goats' milk and goats' milk powder. The published primer was targeted at highly conserved regions in bovine mitochondrial DNA (a 271 bp amplicon). This amplicon was cloned and sequenced to further confirm bovine specific sequence. The chelex-100 was used to separate bovine somatic cells from goats' milk or goats' milk powder samples. Random sampling of different brands of goats' milk powder and tablets from various regions of Taiwan showed the adulterated rate was 20 out of 80 (25%) in goats' milk powders and 12 out of 24 (50%) in goats' milk tablets. With this system, as low as 0.1% cows' milk or cows' milk powder in goat milk or goat milk powder could be identified. This chelex DNA isolation approach provides a fast, highly reproducible and sensitive method for detecting the adulteration of goats' milk products. (**Key Words :** Goats' Milk, Adulteration, Chelex, DNA Extraction)

INTRODUCTION

Goats' milk is a highly nutritious food. It is sold as a nutraceutical food for consumers and costs more than cows' milk. Thus, adulteration of goats' milk, by cows' milk, has become a problem. However, due to their similarity in appearance and composition, it is not easy to differentiate goats' milk from cows' milk. This situation also presents a risk for cows' milk allergy-sensitive people, when consuming adulterated goats' milk.

Currently, several approaches have been taken to identify the adulteration of goats' milk, based on differences in specific protein or lipid profiles, between cows' and goats' milk. The techniques used to estimate these differences include non-immunological methods, analysis of fatty acid composition by gas chromatography or on the detection of specific protein components by HPLC, urea-polyacrylamide gel electrophoresis (CNS, 1998), SDS-PAGE (polyacrylamide gel electrophoresis) (Lee et al., 2004), and isoelectric focusing (Amigo et al., 1991; Kim and Jimeez-Flores, 1993; Chen et al., 2004). All of these methods have the disadvantage of being tedious, time consuming and uneconomic. Immunological approaches use an enzyme-linked immunosorbent assay (Anguita et al., 1996; Ritcher et al., 1997); some false positive and false negative results remain to be overcome. PCR (polymerase chain reaction) provides an alternative way of identifying the additions of foreign milk or dairy products to the original milk by analysis of mitochondrial DNA (Bania et al., 2001; Maudet and Taberlet, 2001; Bottero et al., 2003). Milk secreted from mammary glands contains a number of somatic cells, which include neutrophils and epithelial cells, varying in range from 10^4-10^6 cells/ml in cows' milk; these somatic cells provide a good source of DNA that can indicate the origin of the milk.

The aim of this study was used two pairs (bovine and caprine) of DNA primers to detect cows' milk in goats' milk and goats' milk powder, and to compare the efficiency of DNA isolation from somatic milk cells between the chelex resin and phenol-chloroform extraction methods. Moreover, random sampling different brands of goats' milk powder and tablets from various regions of Taiwan were tested to investigate the prevalence of the adulterated cows' milk in goats' milk powders and tablets, respectively.

MATERIALS AND METHODS

Sample preparation

Saanen goat milk samples were provided by a local goat

* Corresponding Author: Ching-Feng Weng. Institute of Biotechnology, National Dong Hwa University, Hualien, Taiwan, ROC. Tel: +886-3-863 3637, Fax: +886-3-8630255, E-mail: cfweng@mail.ndhu.edu.tw

[2] College of Bioresources, Department of Food Science, National I-Lan University, I-Lan, Taiwan, ROC.

Received December 12, 2005; Accepted March 7, 2006

Table 1. PCR oligonucleotide primers targeting mitochondrial DNA

Species	Position	Accession no.	Ref.
Bovine (271 bp in length)			
5′GCCATATACTCTCCTTGGTGACA 3′ (Forward)	8107/8129	J01394	Tartaglia et al.
5′GTAGGCTTGGGAATAGTACGA 3′ (Reverse)	8377/8357		
Caprine (225 bp in length)			
5′TTAAAGACTGAGAGCATGATA 3′ (Forward)	71/91	AF039171	Lahiff et al.
5′ ATGAAAGAGGCAAATAGATTTTCG 3′ (Reverse)	295/272		

farm. Goats' milk powder was generously donated by the National Laboratories of Foods and Drugs at Taiwan, and commercial products including 4 different brands of goats' milk powder and 3 different brands of goats' milk tablets were purchased in local markets from various regions of Taiwan during 2003-2004. Goat milk tablets after grinding completely in bowl and all commercial goat milk powders were suspended in distilled water and diluted in a ratio of 1:10.6 μl of cows' milk was mixed with 194 μl of goats' milk to make a 3% cows' milk sample in the lab, and a series of mixtures were then diluted by goats' milk at 2, 1, 0.5, and 0.1% for the adulteration test.

DNA extraction

Chelex isolation procedure : The chelex isolation method was modified from previously described (Amills et al., 1997). Briefly, an aliquot of 200 μl goats' milk or cows' milk was subjected to centrifugation at 12,000×g for 2-5 min; the upper layer of butterfat was discarded and the pellet was retained. One ml chelex -100 (prepared from 20% chelex-100 and mixed with 1% triton-×100) was added and mixed gently with the pellets. The mixture was incubated for 15-20 min at 95°C on a dry heat block, then immersed on ice for 1 min and centrifuged at 10,000×g for 5 min. The supernatant was removed for further PCR amplification after determination of its DNA concentration in a spectrophotometer at OD 260/280 nm. This whole process took about 35-45 min.

Phenol-chloroform extraction method : A phenol-chloroform extraction method was used to isolate DNA from the centrifuged pellet. Briefly, the pellet was lysed with 400 μl digestion buffer (0.1 M NaCl, 10 mM Tris-HCl , pH 8.0, 5 mM EDTA in 0.5% SDS), and the mixture was incubated overnight at 37°C with proteinase K (20 mg/ml). Samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) solution. The total DNA, contained in the aqueous phase, was precipitated with 1/10 volume 3 M sodium acetate, pH 5.2, and 2 volumes cold 100% ethanol at 4°C for 30 min. DNA pellets, obtained by centrifugation (13,000×g, 10 min), were washed twice with 70 and 100% ethanol, air dried, and then resuspended in 50 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), with the nucleic acid concentration measured at 260 nm. This procedure took about 12 h. DNA from the meat of goats and cattle was also extracted and used as a positive control.

PCR amplification

Two microliters of the supernatant (template DNA) were added to 23 μl of the PCR mixture, containing 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM of dNTP, 0.25 μM of each primer (forward or reverse) for bovine or carpine milk (Tartaglia et al., 1998; Lahiff et al., 2001; Cheng et al., 2003) (Table 1), and 2 U of Taq DNA polymerase. The primers used in this study were purchased from MDBio, Inc. (Taiwan), and diluted with TE buffer for preparing a stock solution, and stored at -20°C before used. The caprine-specific primers had been tested in four different predominant goat breeds including Sannen, Nubia, Toggenburg and Alpine (Cheng et al., 2003). The PCR was performed in a thermocycler (GeneAmp PCR System 2400, Applied Biosystems, Singapore) under the following conditions: an initial denaturation step at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 30 sec, for 30 cycles, followed by a final extension time at 72°C for 5 min. PCR and negative control extractions were included for each amplification experiment, in order to detect false positives due to contamination. PCR products were examined by electrophoresis, through a 2% agarose (Agarose LE, Promega) gel in 1×TBE buffer (0.045 M Tris-borate, 0.045 M boric acid, 0.001 M EDTA, pH 8.0) and stained by ethidium bromide. A 100 bp DNA ladder marker was used as a size reference.

RESULTS AND DISCUSSION

Nucleic acid-based analysis has been widely used in many fields, and is progressively more admired for identification of feed or food adulterants (Tartaglia et al., 1998; Partis et al., 2000; Lahiff et al., 2001) to warrant the products quality and the purity of ingredient. It seems that the advantages of DNA-based analysis are counted manifold (Partis et al., 2000). First, the ubiquitous DNA, from all cell types of an individual species, contains identical genetic information, regardless of the sample origin. Second, the content of information in DNA is more abundant than that in proteins, due to the degeneracy of the genetic code. Third, DNA is a rather stable molecule that renders its extraction and analysis, from many different

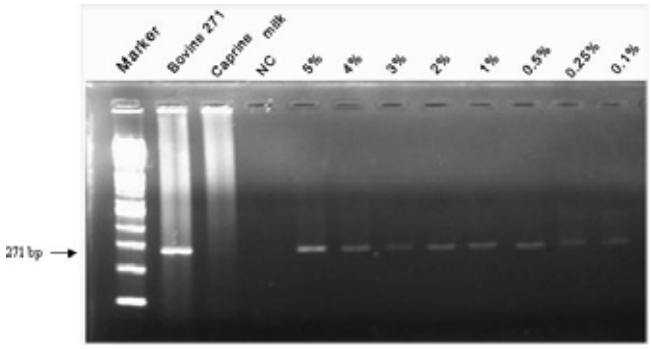

Figure 1. Detection of the inclusions of cows' milk in goats' milk by the chelex quick DNA isolation method. Lane 1: 100 bp DNA ladder marker; lane 2: pure bovine template at 271 bp amplicons; lane 3: pure goats' milk; lane 4: NC (negative control), distilled water only; lane 5-12: representative mixtures of goats' milk containing 5-0.1% cows' milk.

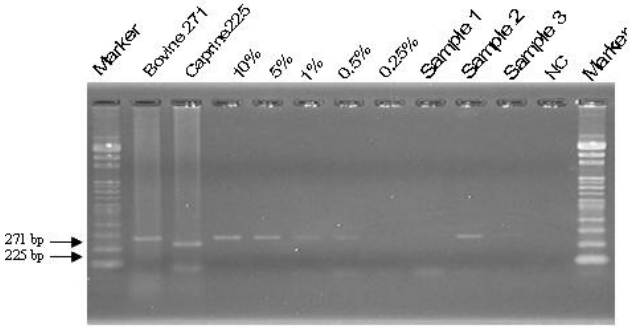

Figure 2. Detection of different inclusions of cows' milk powder in goats' milk powder by chelex quick DNA isolation method. Lane 1 and lane 13: 100 bp DNA ladder marker; lane 2: pure bovine template at 271 bp amplicons; lane 3: pure goats' milk at 225 bp amplicons; lane 4-8: representative mixtures of goats' milk powder containing 10-0.25% cows' milk; lane 9-11: commercial products; lane 12: NC (negative control), distilled water only.

types of samples feasible. A detection method based on mtDNA can improve the sensitivity further, because each cell has only one set of genomic DNA in the nucleus, but bears several copies of mtDNA. There are approximately 1,000 mitochondria in a cell and 10 copies of mtDNA per mitochondrion; 10^4 copies of mtDNA are available per cell, but just one copy for genomic DNA. Therefore, more efficient detection of species-specific DNA results can be obtained by using mtDNA than from using genomic DNA.

Adulteration sensitivity

The present study showed that the oligonucleotide primers targeting bovine and caprine mitochondrial DNA (mt DNA) could specifically amplify for goats' milk and cows' milk, producing 225 and 271 bp amplicons, respectively. The results for goats' milk adulteration by cows' milk are summarized in Figure 1. As little as 0.1% of cows' milk was detected by the chelex quick DNA isolation

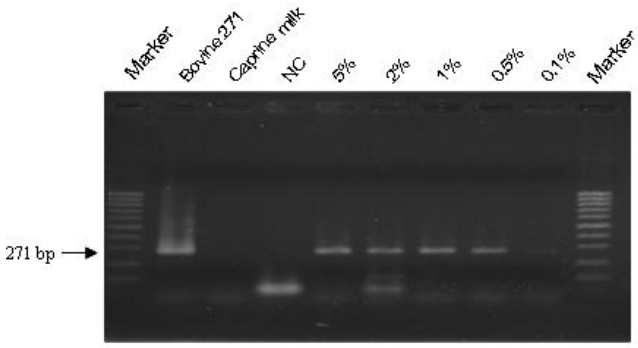

Figure 3. Detection of different inclusions of cows' milk powder in goats' milk powder by the phenol-chloroform extraction method. Lane 1 and lane 10: 100 bp DNA ladder marker; lane 2: pure bovine template at 271 bp amplicons; lane 3: pure goats' milk; lane 4: NC (negative control); lane 5-9: representative mixtures of goats' milk powder containing 5-0.1% cows' milk.

protocol. There were total of 54 milk samples has been tested by this system, and the results showed all samples intent to adulterate above 0.1% were all detected. The phenol-chloroform extraction methods for DNA isolation method had a similar efficiency. The test data for milk powder adulteration are shown in Figure 2. The milk powder was reconstituted by a 10-fold dilution with distilled water, after being mixed thoroughly with pure cows' milk powder. The detection limit was from 1-0.5% for milk powder adulteration, using the chelex quick DNA isolation protocol; the picture showed an obvious band in one of the three commercial goats' milk powder.

Extraction comparison and survey

The results of chelex quick DNA isolation methods (Figure 2) were similar to the phenol-chloroform extraction method (Figure 3), where the detection limit was close to 0.5% of adulteration. The use of the chelex quick DNA isolation protocol, along with a specific bovine primer, was less time consuming for the adulteration survey of commercial labeled goats' milk powder products. However, the banding intensities in Figure 2 were decreasing along with the reduction of inclusions rate of cows' milk powder in goats' milk powder, suggesting the presence of inhibitors. Therefore, the conditions of PCR program and procedure need further optimization. For the samples collected from various regions of Taiwan, the results indicated that there were 20 out of 80 (25% prevalence) goats' milk powders and 12 out of 24 goats' milk tablets (50% prevalence) found to be adulterated with cows' milk powder in Taiwan (Table 2). Only Tai-Nan and Hua-Lien collected goats' milk powders did not show any adulteration. However, all locations collected goats' milk tablets showed the adulteration. We do not know the reason why the adulterated difference between powders and tablets in

Table 2. Adulteration survey in commercial goats' milk products in Taiwan

Sample type	Brand*	Collected location	Samples of adulteration/ total samples	Adulteration (%)
Goats' milk powder	1	I-Lan	3/6	
		Taipei	4/12	
		Tau-Yuan	2/7	
	2	Tai-Chung	3/11	
		Chia-Yi	2/6	
		Tai-Nan	0/7	
	3	Ping-Tung	2/7	
		Hua-Lien	0/5	
	4	Hsin-Chu	2/6	
		Kao-Hsung	2/13	
		Total	20/80	25
Goats' milk tablets	1	Taipei	2/4	
		Tau-Yuan	2/3	
		Kao-Hsung	2/4	
	2	I-Lan	1/2	
		Tai-Chung	1/3	
		Chia-Yi	1/3	
	3	Chang-Hua	2/3	
		Ping-Tung	1/2	
		Total	12/24	50

* The commercial names were labeled with number including 4 different brands of goats' milk powder and 3 different brands of goats' milk tablets collected from various regions of Taiwan during 2003-2004. Tested samples were covered all kind of brands of dairy products, and three independent determinations were done for each sample to exclude the possibilities of false positive.

different location at this moment yet. This fact could be due to the season of goats' milk production or sampling problem. In this study, the sampling time was random and only tested 7 brands of goats' milk products. By applying this chelex DNA isolation approach for detecting the adulteration of goats' milk products needs to be long-term monitored in the future particular in various seasons and more brands of goats' milk products. The major advantage of chelex-based DNA isolation is that it does not require a procedure to purify somatic cells from other milk components, which is tedious and time consuming (CNS, 1998). Furthermore, chelex resin protects the DNA template and Taq polymerase from the damaging effects of the heavy metals ions present in milk, i.e. Zn, Cu, Fe, and Mg. These ionic PCR inhibitors are bound by chelex (Singer-Sam et al., 1989). The total operating time spent for chelex fast detection was only 3 h, which is far less than the national standard regulation method of urea-polyacrylamide gel electrophoresis in Taiwan; including sample preparation and electrophoresis time, the latter method can take up to 7 h. In the phenol-chloroform extraction method, the solution must be freshly prepared and carefully preserved; this is labor-intensive and time consuming. In contrast, when using the chelex method, the same vial was used to carry out all procedures that avoid DNA contamination or carry-over problems, in addition to saving costs. The chelex quick DNA extraction protocol, along with an animal-specific primer, provides a sensitive and time saving approach to identifying cows' milk adulteration in goats' milk products.

CONCLUSIONS

Concluded herein, using chelex-100 was successful in somatic cell DNA extraction. By using this approaches for adulteration survey in Taiwan market, we found goats' milk powder and tablets adulterated rate was 20 out of 80 (25%) in goats' milk powders and 12 out of 24 (50%) in goats' milk tablets, respectively. As low as 0.1% cows' milk or cows' milk powder in goat milk or goat milk powder could be identified in this system. This chelex DNA isolation approach provides a fast, highly reproducible and sensitive method for detecting the adulteration of goats' milk products.

ACKNOWLEDGMENTS

We wish to extend our sincerely thanks to the National Laboratories of Foods and Drugs, in Taiwan, for generously providing partially samples of goats' milk powder and tablets. We also appreciate the support received from the Council of Agriculture in Taiwan.

REFERENCES

Amigo, L., M. Ramos, P. J. Martin-Alvarez and M. Barbosa. 1991. Effect of technological parameters on electrophoresis detection of cow's milk in ewe milk cheeses. J. Dairy Sci. 74:1482-1490.

Amills, M., O. Francino, M. Jansa and A. Sanchez. 1997. Isolation of genomic DNA from milk samples by using chelex resin. J. Dairy Res. 64:231-238.

Anguita, G., R. Martin, T. Garcia, P. Morales, A. I. Haza, I. Gonzalez, B. Sanz and P. E. Hernandez. 1996.. Immunostick ELISA for detection of cow's milk in ewe milk and cheese using a monoclonal antibody against bovine β-casein. J. Food Prot. 59:436-437.

Bania, J., M. Ugorski, A. Polanowski and E. Adamczyk. 2001. Application of polymerase chain reaction for detection of goats' milk adulteration by milk of cow. J. Dairy Res. 68:333-336.

Bottero, M. T., T. Civera, D. Nucera, S. Rosati, P. Sacchi and R. M. Turi. 2003. A multiplex polymerase chain reaction for identification of cows', goats' and sheep's, milk in diary products. Inter. Dairy J. 13:277-282.

Chen, R. K., L. W. Chang, Y. Y. Chung, M. H. Lee and Y. C. Ling. 2004. Quantification of cows' milk adulteration in goats' milk using high-performance liquid chromatography with electrospray ionization mass spectrometry. Rapid Commun. Mass. Spectrom. 18:1167-1171.

Cheng, Y. H., C. M. Wen, S. T. Ding and T. Y. Kuo. 2003. Detecting meat and bone meal in ruminant's feeds by species-specific PCR. J. Anim. Feed Sci. 12:851-860.

CNS. 1998. Method of test for milk and milk products-detection of cow's milk in goat's milk. Chinese National Standard, No.14117, N6304, pp. 1-3.

Kim, H. H. and R. Jimeez-Flores. 1993. Two dimensional analysis of skim milk proteins using preparative isoelectric focusing followed by polyacrylamide gel electrophoresis. J. Food Biochem. 16:307-321.

Lahiff, S., M. Glennon, L. O'Brien, J. Lying, T. Smith, M. Maher and N. Shilton. 2001. Species-specific PCR for the identification of ovine, porcine and chicken species in meat and bone meal (MBM). Mol. Cell. Probe. 15:27-35.

Lee, C. C., H. S. Chang and H. S. Sheen. 2004. A quick novel method to detect the adulteration of cow milk in goat milk. Asian-Aust. J. Anim. Sci. 17:420-422.

Maudet, C. and P. Taberlet. 2001. Detection of cows' milk in goats' cheese inferred from mitochondria DNA polymorphism. J. Dairy Res. 68:229-235.

Partis, L., D. Croan, R. Guo, T. Clark and J. Murby. 2000. Evaluation of a DNA fingerprinting method for determining the species origin of meats. Meat Sci. 54:369-376.

Ritcher, W., I. Krause, C. Graf, I. Sperrer, C. Schwarza and H. Klostermeyer. 1997. An indirect competitive ELISA for the detection of cows' milk and casein in goat's and ewes' milk and cheese using polyclonal antibodies against bovine γ-caseins. Zeitschrift fur Lebensmittel-Untersuchung und-Forschung. 204:21-26.

Singer-Sam, J., R. L.Tanguay and A. D. Riggs. 1989. Use of chelex to improve the PCR signal from a small number of cells. Amplification: A Forum for PCR Users, 3:11.

Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci, and P. A. Battaglia. 1998. Detection of bovine mitochondrial DNA in ruminant feeds: A molecular approach to test for the presence of bovine-derived materials. J. Food Prot. 61:513-518.