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Evaluation of PCR Assay for Detection of Cow's Milk in Water Buffalo's Milk

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Abstract: This study was carried out to evaluate, PCR-based method, for detection of cow's milk in water buffalo's milk. It utilized primers targeting the mitochondrial 12S rRNA gene. The detection limit of the evaluated PCR method was 0.5% and it was determined using model samples made from buffalo's milk containing defined percentages of cow's milk. The method was also evaluated for its applicability for inspection of 21 market milk samples labeled "buffalo milk". Ten out of the 21 examined milk samples were proven to be pure buffalo's milk; three samples were confirmed to be pure cow's milk while the remaining eight samples were mixed cow and buffalo milk. In conclusion, the PCR assays evaluated in this study can be useful for milk inspection to detect cow's milk in water buffalo milk with a detection limit of 0.5%. Also, analysis of market milk samples revealed that adulteration of buffalo milk by mixing with cow's milk or even substitution with cow's milk is a common practice in the dairy field.

Key words: Cow · Buffalo · Milk · DNA · PCR

INTRODUCTION

Recently, species identification of dairy products has received great attention. It has a remarkable importance for several reasons related to governmental regulation, religion and public health. Protection against species substitution or admixture in dairy products is of significant importance [1]. Milk is known to be frequent cause of food allergies. It was found that most milk proteins, even at low concentration, are potential allergens [2,3]. Also, cow's milk was reported as the main dairy product responsible for human adverse reaction [4]. Thus, the counterfeiting of buffalo's milk with cheaper cow's milk may be considered as a health risk making species identification an important issue in current food safety requirement. The common fraudulent practice found in the dairy production line is the use of a cheaper type of milk in substitution of more expensive ones.

In the dairy sector, the fraudulent misdescription of food contents on product labels has been reported especially with high added value milk products commanding a premium price [5]. An outstanding example is the Mozzarella cheese, a typical Italian product that is marketed worldwide, that was originally made from water buffalo's milk. However, similar products are now made using less expensive cow's milk. Species identification of milk used in such products is important, particularly in Mozzarella di Bufala Campana, which is a high grade cheese registered by the European law with the protected designation of origin (PDO) that only made from water buffalo's milk [6].

Currently, different methods are used for species identification in milk and milk products including immunological [7], electrophoretic [8] and chromatographic [9] techniques. Among these methods, capillary electrophoresis, two dimensional electrophoresis, iso-electric focusing of milk caseins which is the European Community reference method for cow's milk detection [10], Also, HPLC and ELISA are reported [11, 12]. However, these methods can't always distinguish milk from closely related species and not suitable for heat treated milk.

Fortunately, molecular techniques have been recently applied for species identification and differentiation and have been proved to be reliable, sensitive and fast. Among molecular techniques, PCR is the most widely used test for the identification of species of origin in milk [1, 6, 13-19].

The objective of this study was to evaluate the PCR assay and its sensitivity and applicability for detection of

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cow's milk in buffalo's milk. Simultaneously, milk analysis of the examined milk was also performed to detect the effect of milk mixing on fat, total solids and solid not fat percentages of milk composition.

MATERIALS AND METHODS

Milk Samples: Five different batches of pure raw milk of both cow and buffalo were collected as standard milk samples from different dairy farms. Milk samples were transported to the laboratory under refrigeration and were processed immediately.

Four independent series of binary mixtures of cow's milk in water buffalo's milk were prepared for further DNA extraction and PCR analysis. For each series, different cow milk percentages containing 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 1 and 0.5%, (v/v) were prepared in a final volume of 50 ml. Also, 21 milk samples obtained from different local milk supermarkets (labeled "buffalo milk") were subjected to PCR analysis to evaluate the applicability of the test for milk samples from the retail trade. All the collected samples were divided into two portions; one was used fresh to be analyzed by Milk analyzer. The other portion was stored at-20°C until time for DNA extraction and PCR.

Extraction of DNA from Milk: Total cellular DNA was extracted from pure, mixed milk mixtures and market milk samples according to the method of Sharm *et al.* [16] with some modifications. Briefly, 470 µl lysis buffer (10 mM Tris-HCL, 100 mM Nacl, 1mM EDTA, pH 8.0 and 0.5% SDS) and 30 µl of proteinase K (20 mg/ml) were added to 200 µl of each milk sample. The mixture was then vortexed and incubated at 37°C overnight. DNA was extracted by equal volumes of Phenol-chloroform-isoamylalcohol (25:24:1) and Chloroform-isoamylalcohol (24:1), successively. DNA was precipitated by adding two volumes of chilled absolute ethanol and one tenth volume of 3M sodium acetate (pH 5.2). The DNA pellet, obtained after centrifugation for 30 min. at 14000 rpm, was washed with 70% ethanol, air-dried and subsequently dissolved in an appropriate volume of double distilled water and quantified by spectrophotometry and diluted to 50 ng/µl.

Primers: Three primers were synthesized using MWG oligosynthesis of MWG Biotech. (Germany) according to sequences reported by Lopez-Callega *et al.* [16]. The first primer is a common forward primer designated as 12SM-FW (5' CTA GAG GAG CCT GTT CTA TAA TCG ATA A 3'). It was reported to be common to both cows and water buffaloes. The second primer is a reverse cow-specific primer designated as 12SBT-REV2 (5' AAA TAG GGT TAG ATG CAC TGA ATC CAT 3'). The 12SM-FW/ 12SBT-REV2 primer pair amplified a 346 bp cow specific DNA fragment. The third primer is a reverse buffalo-specific primer designated as 12SBuf-REV2 (5' TTC ATA ATA ACT TTC GTG TTG GGT GT 3'). The 12SM-FW/12SBuf-REV2 primer pair amplified a 220 bp DNA fragment from water buffalo DNA.

PCR Amplifications: Two different PCR assays were performed [16]. The first one utilized the 12SM-FW/ 12SBT-REV2 primer pair to detect the presence of cow's milk in the samples. The other PCR test utilized the 12SM-FW/12SBuf-REV2 primer pair to detect the presence of buffalo's milk in the samples. All PCR assays were performed in 25 µl reaction volume containing 50 ng of genomic DNA as template, 10 pmol of each primer and 1X of PCR master mix (Taq Master/High yield, Jena Bioscience). The amplification cycles were carried out in a PT-100 Thermocycler (MJ Research, USA). Reaction conditions were optimized to be 93°C for 3 min. as initial denaturation, followed by 40 cycles of 93°C for 30 seconds, 63°C for 30 seconds and 72 °C for 2 min. A final extension step at 72°C for 10 min. was followed. Positive DNA isolated from either pure buffalo or pure cow milks and negative control (no template) were included in each PCR run to ensure no cross contamination or amplification failure due to presence of inhibitors. All tests were repeated twice to ensure reproducibility of the PCR assays.

Agarose Gel Electrophoresis: Amplification products were electrophorezed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 100 bp DNA ladder was run simultaneously as a marker. Presence of 364 bp DNA fragment indicated the presence of cow's milk while presence of 220 bp DNA fragment indicated buffalo milk [16].

Milk Analysis: The fresh portions of milk samples were analyzed by milk analyzer to determine somatic cell count (SCC) and milk composition. SCC was determined at first to exclude any mastitic milk or subclinical mastitic cases using Bently Soma-count 150 (Bentley Instruments Inc., Chaska, MN, USA). Fat, Total solids (TS) and solid not fat (SNF) percentages were determined using the infrared milk analyzer unit Bentley 150 (Bentley Instruments Inc., Chaska, MN, USA).

Statistical Analysis: The effect of mixing milk on fat, total solids (TS) and solid not fat (SNF) percentages of market milk samples was statistically determined by "one way analysis of variance" according to Snedecor and Cochran [21].

RESULTS

In this study, a PCR-based method has been used for the specific detection of cow's milk in water buffalo's milk. Genomic DNA included mitochondrial DNA was successfully isolated from small quantity of all milk samples.

To evaluate the specificity of the primers PCR amplification of cow's milk DNA with the 12SM-FW/12SBT-REV2 primer pair were performed. The expected PCR fragment (346 bp) was amplified in all batches of pure cow's milk, whereas no amplification products were observed with DNA extracted from water buffalo's milk (Fig.1). Also, PCR amplification of buffalo's milk DNA with 12SM-FW/ 12SBuf-REV2 primer pair gave rise to the expected buffalo specific amplicon of (220 bp), whereas no amplification was observed with DNA extracted from cow's milk (Fig. 2).

Fig. 1: PCR products of cow-specific 12S rRNA gene amplified using 12SM-FW/12SBT-REV2 primers and applied on standard milk samples. M, ladder DNA marker, lane 1, buffalo DNA, lanes 2-6 are cow's DNA, NC: negative control 100bp.

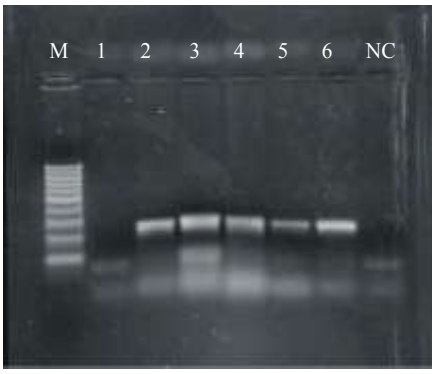

Fig. 2: PCR products of buffalo-specific 12rRNA gene amplified using 12SM-FW/12SBuf-REV2 primers and applied on standard milk samples. M, 100bp ladder DNA marker, lane 1, cow DNA, lanes 2-6 are buffalo DNA, NC, negative control.

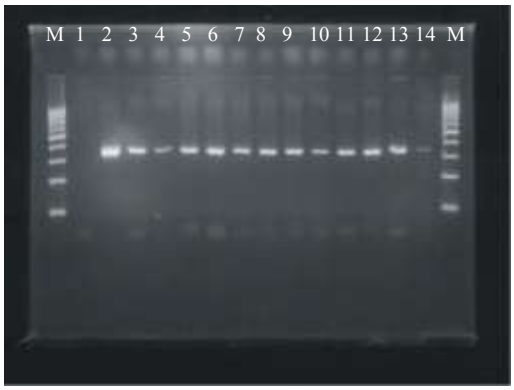

Fig. 3: PCR products of cow-specific 12S rRNA gene obtained from raw milk binary mixtures of cow's milk in buffalo's milk amplified using 12SM-FW/12SBT-REV2 primers. M, ladder DNA marker, lane 1, buffalo DNA, lanes 2-14 are DNA extracted from binary milk mixtures starting from100% down to 0.5% cow's milk in buffalo's milk.

After ensuring the specificity of the selected primers, PCR amplification was performed on binary milk mixtures in order to determine the sensitivity of the PCR assay to detect cow's milk in water buffalo's milk. The results of

Table 1: Fat, total solids and solid not fat in pure and market milk samples (Mean±SE)

	Pure milk		Market milk samples		
	Buffalo	Cow	Group I	Group II	Group III
Fat	7.07±0.21^{a}	3.92±0.16^{bc}	4.91±0.23^{b}	2.77±0.75^{c}	4.61±0.32^{b}
TS	16.3±0.33^{a}	12.33±0.17^{b}	13.64±0.37^{b}	11.41±0.59^{b}	13.25±0.74^{b}
SNF	9.23±0.40	8.41±0.16	8.73±0.20	8.66±0.40	8.22±0.33

$P < 0.05$,

Means with different alphabetical letters in the same row are significantly differed

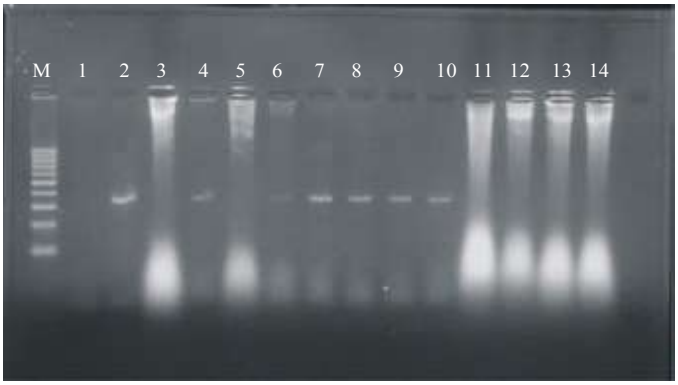

Fig. 4: PCR products of cow-specific 12S rRNA gene obtained from market milk samples amplified using 12SM-FW/12SBT-REV2 cows-specific primers. M, ladder DNA marker, lane 1, negative control, lanes 2,4,6,7,8,9,10 showed positive amplification of 364 bp cow specific PCR products. Lanes 3,5,11,12,13,14 showed no amplification.

Fig. 5: PCR products of buffalo-specific 12S rRNA gene obtained from market milk samples amplified using 12SM-FW/12SBuf-REV2 buffalo-specific primers. M, ladder DNA marker, lane 1,buffalo positive DNA, lanes 2,4,5,6,7,8,9,10, 12,13,15 showed positive amplification of 220 bp buffalo-specific. Lanes 3, 11,14 are negative samples.

PCR amplification performed using the 12SM-FW/ 12SBT-REV2 primer pair showed a consistent PCR amplification of cow 346 bp DNA fragment from milk, with a detection threshold of 0.5% as shown in Figure 3. Results of PCR amplification on four independent series of milk mixtures prepared with four different batches of pure cow's and buffalo's milk revealed the same detection limit.

To evaluate the applicability of the assay, PCR amplification using 12SM-FW/ 12SBT-REV2 primer pair was used for detection of cow's milk in purchased market milk samples (labeled Buffalo milk). Amplification of the cow specific 346 bp amplicon indicated the presence of cow's milk in some milk sample as seen in Figure 4. Out of the 21 tested samples, 11 samples contained cow's milk. In order to verify the presence of buffalo milk DNA, a parallel PCR control assay utilizing 12SM-FW/12SBuf-REV2 primers was performed on the same samples. Amplification of buffalo specific 220 bp amplicon fragment verifies the presence of buffalo milk (Fig. 5). Combining the results of both tests confirmed 10 samples to be pure buffalo's milk, 3 samples to be pure cow's milk, while the remaining 8 samples were mixed milk. All the results were reproducible when performed twice.

In parallel to the PCR assays, milk analysis was carried out on standard buffalo's, cow's and market milk samples to determine fat, total solids (TS) and solid not fat (SNF) percentages. Market milk samples were divided into 3 groups according to their PCR results. Group I confirmed pure buffalo's milk; group II confirmed pure cow's milk and group III confirmed mixed milk.

DISCUSSION

Accurate species identification by PCR is highly dependent on the specificity of primers used. These primers should target a DNA segment with sufficient species to species variation.

The present PCR assays involved the use of three different primers previously developed by Lopez-Calleja *et al.* [16]. A reverse primer specific for cow (12SBT-REV2) was designed complementary to the gene fragment of 12S rRNA. Differences between cow and other ruminants were remarkably in this gene fragment. This cow specific primer, along with the common forward primer (12SM-FW), was expected to yield a cow specific amplicon of 346 bp in the 12S rRNA gene. On the other hand, a buffalo specific primer (12SBuf-REV2) along with the same common forward primer (12SM-FW) was expected to yield a buffalo specific amplicon of 220 bp fragment in the same gene. These primers were chosen because they targeted the mitochondrial encoded gene for 12S rRNA as the target for species identification. These non-nuclear targets possess several advantages over nuclear genes [22]. They are generally more abundant in any given sample than any single-copy nuclear genes. Also, mitochondrial DNA tends to be inherited through the maternal germ line and the resulting lack of heterozygosity in the alleles simplifies analysis [23]. Its advantage over the methods utilizing single primer pair is the elimination of false negative results. At first, genomic DNA included mitochondrial DNA from milk samples was extracted using the method described by Sharma *et al.* [20] and modified later on by Abdel-Rahman *et al.*[17]. The method was used successfully to extract DNA from small quantity of milk samples.

To ensure the specificity of the primers, PCR amplification of cow's milk DNA with 12SM-FW/12 SBT-REV 2 primer pair was performed on all batches of pure milk. The results indicated the specificity of this primer pair for cow's milk only whereas no amplification was observed with water buffalo's milk DNA. Also, PCR amplification of buffalo's milk DNA with 12 SM-FW/ 12 SBuf-REV2 primer pair was performed on all batches of pure milk. Results indicated the specificity of this primer pair for buffalo's milk only whereas no amplification was observed with cow's milk DNA. Also, it was necessary to determine the detection limit of this PCR assay before stating that it can be reliably used for detection of undeclared quantity of cow's milk in water buffalo's milk. PCR amplifications were performed on binary milk mixtures prepared for determining the detection limit. These binary milk mixtures were subjected to DNA isolation and PCR amplifications.

To obtain better sensitivity, optimization of PCR was performed. The duration of the elongation step was found to be important for generation of amplicons. Two minutes elongation step allowed the detection of down to 0.5% cow's milk in water buffalo's milk. This detection limit was verified in all the independent series of milk mixtures ensuring reproducibility of the results. According to the ECR [10, 24], 1% is considered the minimum limit to state the presence of undeclared cow's milk in water buffalo's milk. The obtained 0.5% detection limit in this study was well in keeping with the results of other literature. PCR helped to detect addition of 1% cow's milk in buffalo's milk [6] and 5% [25]. Mitochondrial 12S, 16S rRNA genes based method detected 0.1% addition of cow's milk in sheep's and goat's milk [15]. Cozzolino *et al*. [26] considered 5% detection limit as sufficient for the proof of undeclared milk component, whereas adulteration of milk by less than 5% lacks any economic effect. Also, they stated that although the ability to detect lower levels of contaminating milk could be interesting, but in fact it could be difficult to establish if a fraud is presumable or it just unintentional contamination might be supposed.

After evaluation of the specificity and sensitivity of the primers was established, its applicability on milk samples from retail market was also evaluated. PCR amplification using 12SM-FW/ 12SBT-REV2 primer pair was used for detection of cow's milk in 21 purchased market milk samples. Amplification of the cow specific 346 bp amplicon indicated the presence of cow's milk in 11 milk samples. In order to verify the presence of buffalo's milk DNA, a parallel PCR control assay utilizing 12SM-FW/12SBuf-REV2 primers was performed on the same samples. Amplification of buffalo specific 220 bp amplicon fragment verifies the presence of buffalo's milk in 18 milk samples. The outcome of both PCR assays indicated that 10 samples were pure buffalo's milk, 3 samples were pure cow's milk while the other 8 samples were mixed cow and buffalo milk. These results indicated the applicability of the assay for inspection of market milk.

In parallel to PCR assays, milk analysis was also performed on standard buffalo's, cow's and the 3 groups of market milk samples as divided according to the PCR results to determine fat, TS and SNF percentages. Results of milk analysis of both pure standard buffalo's and cow's milk were compared with the values of EOSQM [27] for milk. It revealed normal values of all milk parameters including fat, protein, SNF and TS percentages. On the other hand, milk analysis of the three different groups of the purchased milk samples, as divided by PCR result, revealed the following: In group I, which is confirmed to

be pure buffalo's milk by PCR, the mean values of milk fat, SNF and TS % were 4.91± 0.23, 8.73±0.20 and 13.64±0.37%, respectively. These results were lower than that of standard pure buffalo's milk. This could be attributed to milk adulteration either by addition of water or partial skimming, but not by mixing with cow's milk [28, 29]. In group II, which is confirmed to be pure cow's milk by PCR, the mean value of fat % was 2.77±0.75 which was lower than the legal fat % of cow's milk. Additionally, SNF% was 8.66±0.40 which is in the normal range of standard cow's milk. Lowering of fat % with normal SNF%, indicated little degree of adulteration by partial skimming only [28, 29]. In group III, which was confirmed to be mixed cow's and buffalo's milk by PCR, the mean values of fat, SNF and TS% were 4.61±0.32, 8.22±0.33 and 13.25±0.74, respectively. All these values were lower than that of standard buffalo's milk that indicated milk adulteration by both partial skimming and mixing buffalo's milk with cow's milk as confirmed by PCR.

CONCLUSION

The PCR assays reported in this study can be useful for milk inspection to detect cow's milk in water buffalo milk with a detection limit of 0.5%. Adulteration of buffalo's milk by addition of cow's milk or even substitution with cow's milk is a common practice in the market.

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