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Comparison of PCR Methods for Determination of Different Types of Milk Added to Goat Milk

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ABSTRACT

Objective: This study, it was aimed to determine which of the multiplex conventional PCR and Real-Time PCR methods are more suitable for the detection of cow and sheep milk mixed with goat milk. **Materials and Methods:** For this purpose, one liter of each goat, cow, and sheep milk was obtained from farms in Van province. PCR experiments were carried out by adding cow's milk and sheep's milk in the same proportions into goat milk (1%, 2%, 5%, 0.1%, and 0.5%). Multiplex conventional and Real-Time PCR were used in these trials. **Results:** In the cow and sheep milk trials, it was determined that the presence of 1%, 2%, and 5% cow and sheep milk added to goat milk could be determined by the multiplex conventional PCR method. However, it was observed that the positivity of the gel image of the milk mixtures added at the rate of 0.5% was unclear, and the mixtures at the rate of 0.1% could not be detected. In the Real-Time PCR method, the presence of cow and sheep milk was detected in all the mixtures and positive graphics were determined. **Conclusion:** This showed that the Real-Time PCR method gives more reliable results even when 0.1% cow or sheep milk is mixed with commercially available goat milk.

Keywords: Goat milk, Multiplex conventional PCR, Real Time PCR.

Keçi Sütüne Eklenen Farklı Süt Türlerinin Belirlenmesi İçin PCR Yöntemlerinin Karşılaştırılması

ÖZ

Amaç: Bu çalışma, keçi sütüne karıştırılmış inek ve koyun sütünün tespiti için multiplex konvansiyonel PCR ve Real Time PCR yöntemlerinden hangisinin daha uygun olduğunun belirlenmesi amacıyla yapılmıştır. **Gereç ve Yöntem:** Bu amaçla Van ilindeki çiftliklerden keçi, inek ve koyun sütünden birer litre süt temin edilmiştir. Keçi sütüne aynı oranlarda inek sütü ve koyun sütü 0.5%, 0.1%, 1%, 2%, 5%) ilave edilerek PCR deneyleri yapılmıştır. Bu denemelerde mültipleks konvansiyonel ve Real Time PCR kullanıldı. **Bulgular:** İnek ve koyun sütü denemelerinde keçi sütüne ilave edilen %1, %2 ve %5 inek ve koyun sütünün varlığının multipleks konvansiyonel PCR yöntemi ile belirlenebileceği ortaya konmuştur. Ancak %0.5 oranında eklenen süt karışımlarının jel görüntüsünün pozitifliğinin belirsiz olduğu ve %0.1 oranındaki karışımların tespit edilemediği görülmüştür. Real Time PCR yönteminde ise tüm karışımlarda inek ve koyun sütü varlığı tespit edildi ve pozitif grafikler belirlendi. **Sonuç:** Bu sonuçlar, Real Time PCR yönteminin, ticari olarak satılan keçi sütü ile %0.1 inek veya koyun sütü karıştırıldığında bile daha güvenilir sonuçlar verdiğini gösterdi.

Anahtar Kelimeler: Keçi sütü, Multiplex konvansiyonel PCR, Real Time PCR.

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INTRODUCTION

Goat milk, it contains less trans fatty acids than cow's milk, it is easier to digest and has less allergic effects. Since a large amount of milk can be obtained from goat breeds with high milk yield, goat breeding and goat milk production are increasing in our country and in the world. Accordingly, consumers tend to buy more goat milk and its products (Paszczyk and Łuczyńska, 2020).

Depending on the seasons, the production of goat milk mostly by families engaged in farming and small-scale enterprises and the increasing demand of consumers for goat milk cause this milk to be offered for sale at higher prices compared to cow and sheep milk. For this reason, it has come to the fore that the milk of different kinds of animals, especially cow's milk, can be mixed with goat's milk, since it is cheaper and more plentiful. If this situation is not reported on the label of the product, it is considered as adulteration and is prohibited according to the legislation of many countries (Golinelli et al., 2014; Alikord et al., 2018).

It is reported that the information on the labels of some foods in the world does not reflect the truth, and it is stated that such practices negatively affect food safety (Di Pinto et al., 2017). Food labels should enable consumers to make informed choices about the products they buy and should always contain accurate information (TFC, 2017). In addition, illegal trade should be avoided and the origin of the milk types should be verified so that unfair profits can be prevented (Golinelli et al, 2014; TFC, 2017). Species detection in dairy products has recently attracted great interest, as the identification of species substitutes or mixtures is important for consumer protection and public health (Bottero et al., 2009). In Türkiye, the Turkish Food Codex Communiqué on Drinking Milk (TFC, 2019) states that the labels of other milks, excluding cow's milk, which are offered to the market, should include the product name and the information from which animal the milk is obtained from.

Many analytical methods, including immunological approaches have been developed to determine which animal species the milk belongs to (Abbas et al., 2018). These methods are chemical, Enzyme-Linked Immunosorbent Assay, (ELISA) (López-Calleja et al., 2007; Stănciuc (Sava) and Râpeanu, 2010; González-Martínez et al., 2018), Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC) (Ten-Domenech et al., 2015), Urea Polyacrylamide Gel Electrophoresis (Urea PAGE) (Duarte-Vázquez, 2018), Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Ma et al., 2019) and Isoelectric Focusing Methods (Chen et al., 2004).

Electrophoretic methods based on differences in chromatic and protein profiles have disadvantages such as time consuming and not being economical (Alikord et al., 2018). The polymerase chain reaction (PCR) method on the other hand, is used as an alternative method to determine whether different milk or dairy products have been added to the examined milk (Bottero et al., 2003; López-Calleja et al., 2004; Cheng et al., 2006; Mašková and Paulíčková, 2006; Rodrigues et al., 2012; Di Pinto et al., 2017). The PCR method is a fast and sensitive method used for determination and verification of milk type, and it is also the method with the lowest margin of error (Cosenza et al., 2019). In addition, DNA-based methods such as PCR have also been applied to ripened cheeses and heated dairy products, compared to protein-based methods that are not always applicable and must be carefully selected (López-Calleja et al., 2007; Stănciuc (Sava) and Râpeanu, 2010; Agrimonti et al., 2015; Kara et al., 2016).

Although the European Union proposes protein-based methods for species identification (CR, 2001), nucleic acid-based techniques have been used instead of protein for species identification, especially in foods of animal origin (Kumari et al., 2015). Among these techniques, PCR, multiplex PCR, Restriction Fragment Length Polymorphism (RFLP), PCR-RFLP and Real Time PCR are the most widely used molecular techniques (Bottero et al., 2003; Natonek-Wiśniewska and Krzyścin, 2019).

With the PCR method, which is among the DNA-based technologies, small amounts of DNA can be amplified quickly and specifically (Rodríguez-Ramírez et al., 2011). In addition, since the gene regions targeted by PCR are relatively small, DNA molecules degraded by the thermal, chemical and/or physical processes in which the food is prepared can also be detected (Drummond et al., 2013).

Complicated mixes can be determined in a single step when using the multiplex conventional PCR method, provided that the specific amplicons are of different lengths and are readily determined by agarose gel electrophoresis. Multiplex conventional PCR method can be applied for the separation of cow, goat, sheep and buffalo milk (Bottero et al., 2003). However, conventional techniques allow the qualitative detection of different species in the presence of a defined detection limit. On the other hand, in the Real Time PCR method, the identification of species is quantitative and this method has more sensitivity (Natonek-Wiśniewska and Krzyścin, 2019).

This research study was carried out to determine which of the multiplex conventional and Real Time PCR methods is more suitable for the detecting of cow and sheep milk mixed with goat milk.

MATERIALS AND METHODS

Material

In this study, cow, sheep and goat milk (one liter each) was obtained from farms in Van. Cow and sheep milks were added to goat milk at the rates of 0.5%, 0.1%, 1%, 2%, and 5%, respectively.

Method

DNA extraction

Commercial kit (GeneAll, ExgeneTM Cell SV, South Korea) was used for DNA extraction from milk. Pure DNA samples obtained in accordance with the manufacturer's recommendations were stored at -20±1 °C until the analysis.

Multiplex conventional PCR method

In this research, specific primers for goat, sheep and cow species were used, which target 12 and 16 mitochondrial rRNA and designed by Bottero et al. (2003) (Table 1).

Ready commercial master mix (abm 2X PCR Taq Plus Master Mix, Canada) was used for the multiplex conventional PCR analysis used to determine the DNAs of the species. In the preparation of PCR mixes, 1 µl (10 µM) of each primer and 5 µl of genomic DNA were added to the 10 µl master mix and the total volume was completed to 25 µl with PCR water. For DNA amplification of the PCR mixture created for the determination of cow and sheep milk in goat milk, the DNA amplification was carried out by a thermocycler (Qiagen Rotor-Gene Corbett Research, USA) with an initial denaturation of DNA at 94 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 30 sec., annealing at 58 ℃ for 60 sec., extension at 72 ℃ for 60 sec., followed by a final extension at 72 ℃ for 5 min. Multiplex conventional PCR analysis was performed in five replications.

Agarose gel electrophoresis

Gel-red (abm, Safe View ClassicTM G108, Canada) stained 1.5% agarose gel was prepared for gel electrophoresis of amplicons obtained as a result of multiplex conventional PCR process. Specific DNA and positive control bands obtained with the help of DNA marker were observed in the gel imaging device (Genesis®, England).

Table 1. Oligonucleotides used as multiplex conventional PCR primers.

Species and genes	Oligonucleotide primers	bp
Goat (*Capra hircus*) M55541[a]	Sens 144 F: 5'CGCCCTCCAAATCAATAAG 3' Antisens 469 R:5'AGTGTATCAGCTGCAGTAGGGTT3'	326 bp
Sheep (*Ovis aries*) NC001941[a]	Sens959 F:5'ATATCAACCACACGAGAGGAGAC 3' Antisens 1130 R: 5'TAAACTGGAGAGTGGGAGAT3'	172bp
Cow (*Bos taurus*) NC001567[a]	Sens 916 F: 5'GTACTACTAGCAACAGCTTA 3' Antisens 1171 R: 5'GCTTGATTCTCTTGGTGTAGAG3'	256 bp

a GenBank accession number

Real Time PCR method

In order to determine the DNA of the species, a commercial kit (DIAGEN 2103, 2104, 2110, Türkiye) was used for Real Time PCR analysis. For this purpose, PCR mix consisting of 10 µl mix A, 5 µl mix B and 5 µl DNA of each species was prepared in line with the manufacturer's recommendations. The DNA amplification was carried out by a thermocycler (Qiagen Rotor-Gene Corbett Research, USA) with an initial denaturation of DNA at 95 ℃ for 5 min followed by 35 cycles of denaturation at 95 ℃ for 10 sec., annealing at 59 ℃ for 30 sec., extension at 72 ℃ for 5 sec., followed by a final extension at 25 ℃ for 1 min. Real-time PCR analysis was performed in five replications.

Ethical consideration

Ethics committee approval is not required as no living material was used in the study.

RESULTS

The results of multiplex conventional PCR analysis of the samples added to goat milk at the rate of 0.5%, 0.1%, 1%, 2%, and 5%, cow's milk and also sheep's milk in the same proportions are shown in Figures 1 and 3, and also Real Time PCR analysis results are shown in Figures 2 and 4.

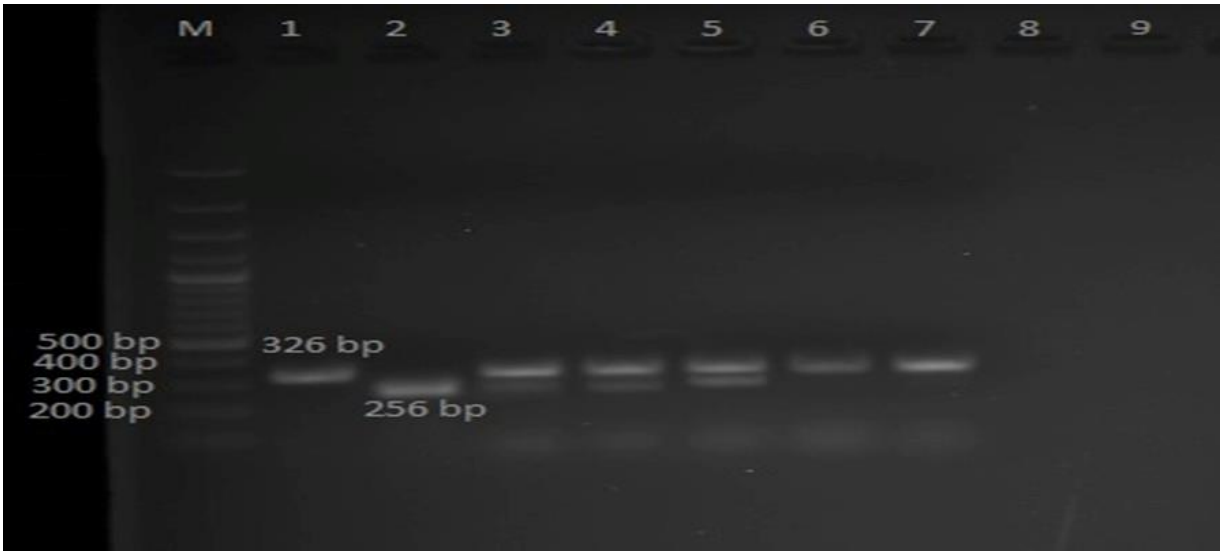

Fig 1. The image of the presence of cow's milk obtained by multiplex conventional PCR method in experimentally prepared goat milk. (M: 100 bp marker; 1: Goat milk (%100) (326 bp); 2: Cow milk (%100) (256 bp); 3: Goat and cow milk (1%) mix; 4: Goat and cow milk (2%) mix; 5: Goat and cow milk (5%) mix; 6: Goat and cow milk (0.1%) mixture; 7: Goat and cow milk (0.5%) mix; 8: Goat primer + cow milk DNA; 9: Cow primer + goat milk DNA)

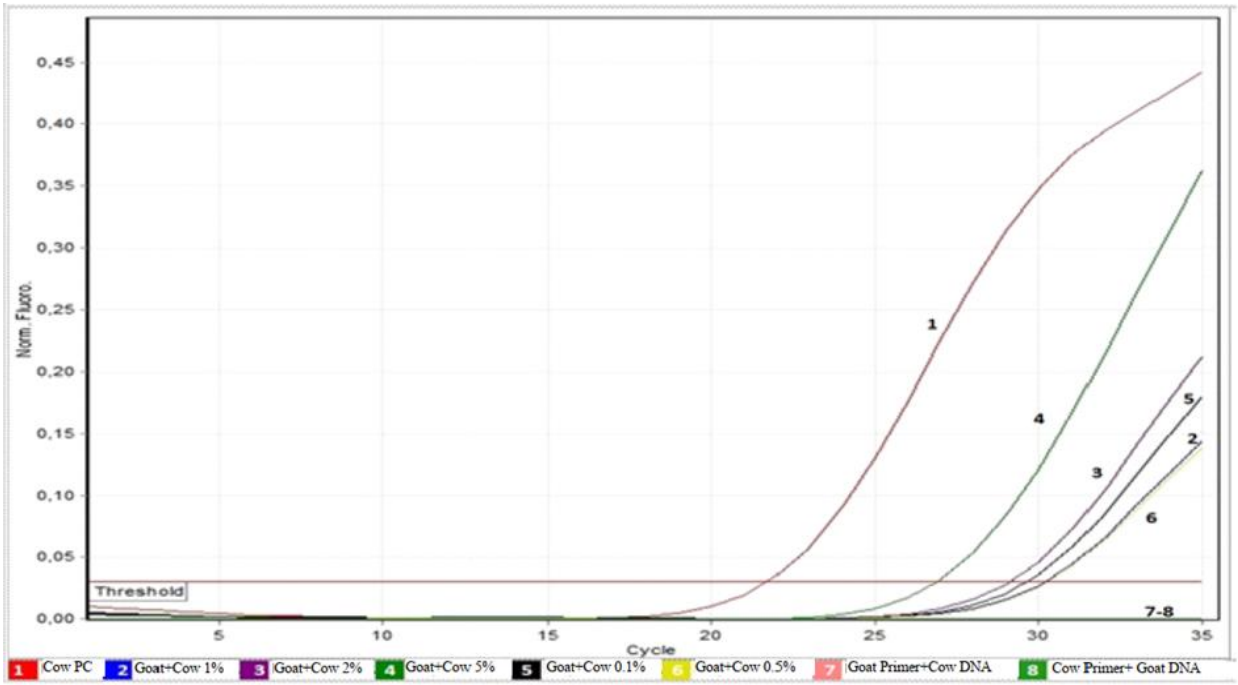

Fig 2. The image of the presence of cow's milk obtained by Real Time PCR method in experimentally prepared goat milk. (1: Cow's milk PC; 2: Goat and cow milk (1%) mix; 3: Goat and cow milk (2%) mix; 4: Goat and cow milk (5%) mix; 5: Goat and cow milk (0.1%) mixture; 6: Goat and cow milk (0.5%) mix; 7: Goat primer + cow milk DNA; 8: Cow primer + goat milk DNA).

Ct values of Real Time PCR results obtained from goat and cow milk mixtures are given in Table 2, and Ct values of Real Time PCR results obtained from goat and sheep milk mixtures are given in Table 3.

Table 2. Ct values of Real Time PCR results obtained from goat and cow milk mixtures.

No.	Colour	Name	Type	Ct
1		Cow milk	Positive Control	21.73
2		Goat's milk + 1% cow's milk	-	30.20
3		Goat's milk + 2% cow's milk	-	29.15
4		Goat's milk + 5% cow's milk	-	26.90
5		Goat's milk + 0.1% cow's milk	-	29.68
6		Goat's milk + 0.5% cow's milk	-	30.29
7		Goat primer + cow milk DNA	Negative Control	
8		Cow primer + goat milk DNA	Negative Control	

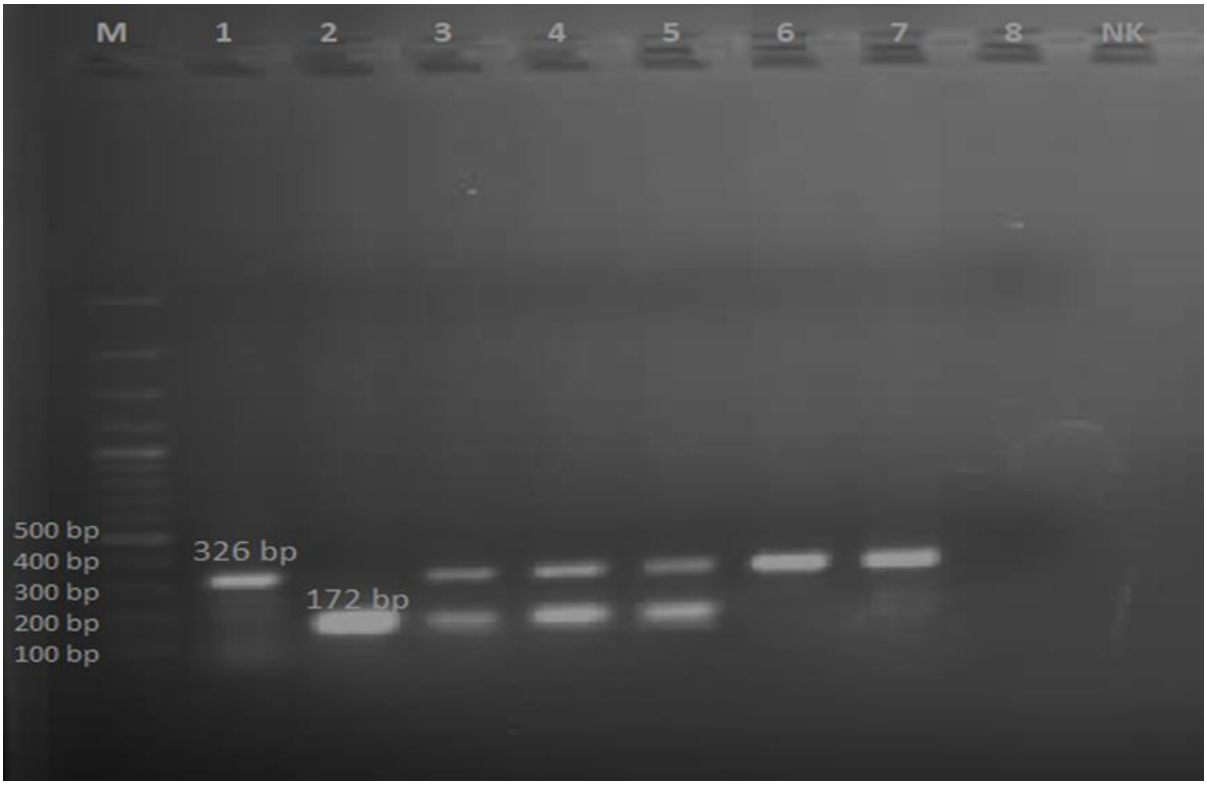

Figure 3. The image of the presence of sheep milk obtained by multiplex conventional PCR method in experimentally prepared goat milk. (M: 100 bp marker; 1: Goat milk (%100) (326 bp); 2: Sheep milk (%100) (172bp); 3: Goat and sheep milk (1%) mix; 4: Goat and sheep milk (2%) mix; 5: Goat and sheep milk (5%) mix; 6: Goat and sheep milk (0.1%) mixture; 7: Goat and sheep milk (0.5%) mixture; 8: Goat primer + sheep milk DNA; 9: Sheep primer + goat milk DNA).

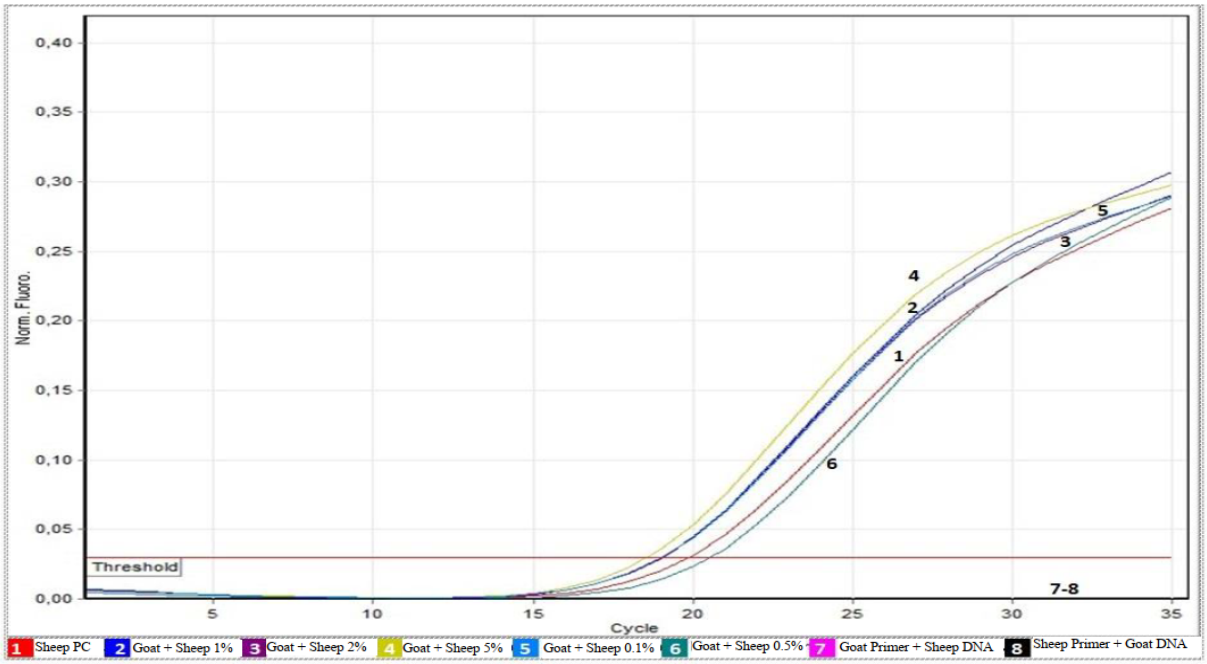

Fig 4. The image of the presence of sheep milk obtained by Real Time PCR method in experimentally prepared goat milk. (1: Sheep milk PC; 2: Goat and sheep milk (1%) mix; 3: Goat and sheep milk (2%) mix; 4: Goat and sheep milk (5%) mix; 5: Goat and sheep milk (0.1%) mixture; 6: Goat and sheep milk (0.5%) mixture; 7: Goat primer + sheep milk DNA; 8: Sheep primer + goat milk DNA).

Table 3. Ct values of Real Time PCR results obtained from goat and sheep milk mixtures.

No.	Colour	Name	Type	Ct
1		Sheep milk	Positive Control	19.87
2		Goat's milk + 1% sheep's milk	-	19.05
3		Goat's milk + 2% sheep's milk	-	19.05
4		Goat milk + 5% sheep milk	-	18.57
5		Goat milk + 0.1% sheep milk	-	19.02
6		Goat milk + 0.5% sheep milk	-	20.54
7		Goat primer + sheep milk DNA	Negative Control	
8		Sheep primer + goat milk DNA	Negative Control	

DISCUSSION

Milk, which contains most of the nutrients in sufficient amounts and is considered the closest food to perfection, has an important place in the nutrition of individuals of all ages (FDA, 1995). Many proteins in milk and dairy products are potential allergens and have a significant effect on the formation of food allergies. In addition, cow's milk has been reported to be a dairy product responsible for the adverse reaction (Rance et al., 2005). Species definitions in milk and dairy products are important in terms of preventing health risks that may occur in people who are sensitive to some dairy products and ensuring food safety. However, in commercial milk production, the use of cheaper milk instead of high quality and costly milk is a common practice that deceives the consumer (Khatun et al., 2021). For these reasons, the use of analytical methods that give fast and accurate results in the analyses and adequate inspections will contribute to the protection of public health (Derinöz et al., 2021). In this study, the detection limit was determined as 1% in the multiplex conventional PCR method. Cheng et al. (2006), in their study with the PCR method, stated that they found the detection limit in goat milk powder to be 0.5-1% after adding 0.5%, 0.1%, 1% and 2% cow's milk into the goat milk

powder. Mašková and Paulíčková (2006), in their study on the determination of the presence of cow's milk in goat and sheep cheeses using the multiplex PCR method, reported that cow's milk was found in 3 of 17 goat cheeses and 1 of 7 sheep cheeses, and the detection limit in the study was determined as 1%. It is seen that the detection limits determined in different studies are similar to the detection limits determined in this study. Bottero et al. (2003) reported that the minimum detection limit was determined as 0.5% in the multiplex PCR method they applied for species identification in the curd cheese they produced by adding 1%, 0.5% and 0.1% cow's milk to goat milk. In a study by López-Calleja et al. (2004), in which cow's milk (0.5%, 0.1%, 1.5%, 10% and 100%) was added to sheep and goat milk to identify species, the multiplex PCR method was used to determine the percentage of different milks in raw and heat-treated milk and dairy products, it has been stated that it can be detected at a rate of 0.1%. Rodrigues et al. (2012) reported that the detection limit was 0.5% in their study by adding cow's milk at 0%, 0.1%, 0.5%, 1%, 5%, 10%, 50% and 100% concentrations to fresh goat milk to determine the analytical sensitivity of the duplex PCR method. It is thought that the differences between the studies may have arisen from the devices, consumables, materials and methods used in the analysis. In this study, the detection limit was determined as 0.1% in the Real Time PCR method. Agrimonti et al. (2015), in their study using the quadruplex PCR (qxPCR) test to detect cheats in dairy products, added buffalo, sheep and goat milk to cow's milk at a rate of 0.1%, 0.5%, 1%, 1%, 1% and 25%. They reported that they found the qxPCR detection limit to be 0.1% in their study. In this study, the detection limit determined by the Real Time PCR method in milk is similar to the findings of different studies. Di Pinto et al. (2017) reported that 58 of the 80 goat milk products they examined using the end-point PCR method were not compatible with the information specified on the labels. In the same study, they stated that both Real Time PCR results confirmed the end-point PCR results and 64 out of 80 products examined according to the Real Time PCR method were inconsistent with the information specified on the labels. The fact that Di Pinto et al. (2017) reported that the Real Time PCR method gave more sensitive results is in line with the findings of this study. In addition, some other researchers (Drummond et al., 2013) have also stated that the Real Time PCR method has more sensitivity.

CONCLUSION

As a result of this research, it was determined that the presence of cow and sheep milk (at the rate of 1%, 2% and 5%) added to goat milk could be detected by multiplex conventional PCR method. However, it was observed that the positivity of the gel image of the milk mixtures added at the rate of 0.5% was unclear, and the mixtures at the rate of 0.1% could not be detected. The high detection limit in the multiplex conventional PCR method based on gel electrophoresis makes it difficult to determine the different milks mixed into the milk in very small amounts. Conventional techniques allow only qualitative detection of different types of milk with a defined detection limit. In addition, the fact that this method requires more than one repetition while optimizing the temperature and time caused wastage of time and consumables. In the Real Time PCR method, the presence of cow and sheep milk was detected in all of the mixtures (1%, 2%, 5%, 0.5% and 0.1%) and positive graphics were determined. This showed that the presence of even 0.1% cow and sheep milk in commercially available goat milk can be detected more reliably by the Real Time PCR method. The lower detection limit in the Real Time PCR method can be attributed to the originality and higher sensitivity of this method. As a result, it has been seen that the detection limit is high in the multiplex conventional PCR method, and it requires more than one repetition while optimizing the temperature and time, which causes waste of time and consumables. In the Real Time PCR method, it has been determined that the detection limit is lower, even cow and sheep milk, which is added to goat milk at very low rates, can be determined and fast results can be obtained in a short time. For these reasons, it has been concluded that the Real Time PCR method is a fast and effective method that can be used routinely for the detection of cow and sheep milk mixed with goat milk.

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Conflict of Interest

The author declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Author Contributions

Plan, design: RMT, YCS; **Material, methods and data collection:** RMT; **Data analysis and comments:** RMT, YCS; **Writing and corrections:** RMT, YCS.

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