



DNA-based analytical methods for milk authentication

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Abstract

Milk adulteration is a globally upcoming problem that concerns consumers, food industries and control authorities. Milk authenticity testing of dairy products is necessary, to protect consumers from fraudulent products, mislabeling and health risks and avoid unfair competition of food industries. Milk-based products produced by milk from specific animal origin are considered as healthier dairy products compared to others, leading to widespread acceptance of the consumers and food industries. This has made these animal-originated products extremely vulnerable to substitution with cheaper milk for economic profit. In addition, milk derived from specific animals (e.g., bovine) has been accused for allergenicity issues and other health problems, especially to sensitive human groups. Therefore, there is a great need for sensitive, accurate and quantitative analytical methods for milk adulteration detection in raw and processed milk-based products.

Keywords Nucleic acids · DNA amplification · Dairy products · Milk · Food authentication · Food adulteration

Introduction

Food adulteration has recently become a very important emerging problem worldwide, due to the rapid increase of food overconsumption, alongside with the market globalization. This issue is attracting increasing interest by the consumers who are eventually concerning about the food origin, safety, authenticity and quality. For this reason, the proper labeling of raw, as well as processed food products is of major demand nowadays. A considerable number of food products with a high added value are extremely vulnerable to adulteration with related products with much lower cost and nutritional value for economical profit [1–4]. In addition to the lower nutritional benefits of the adulterated products, safety issues are also a sincere concern because some adulterants may also be severely health hazards causing serious allergenic events or induce atopy, intolerance and inflammatory diseases [5–10]. This reinforces the criticalness of the food adulteration problem and increases strongly the need for new and sensitive analytical methods for food traceability and authenticity tests.

Dairy products are one of the seven more vulnerable foods to fraudulent adulteration. The extent, though, of the adulteration varies within the countries [1, 11]. Specific milk-based products, such as the ones derived from donkey, goat or sheep are reputed as healthier and of higher quality and nutritional value particularly from bovine milk, leading to a widespread acceptance of the consumers [12]. It has been reported in many cases that in a variety of dairy products of various animal origin, the milk has been replaced totally or partially with cheaper milk, e.g. bovine, to earn more profit. Therefore, there is a great need for the detection of these substitutes or admixtures to protect the consumers from fraud and misleading labeling, as well as from health hazards. Moreover, milks from specific origin, such as bovine milk, has been proved to be responsible for food allergenicity caused mainly in children and other health issues [13]. Cow milk proteins are among the most common food allergens in IgE- and non-IgE-mediated food-allergic disorders in children [14]. Thus, attempts should also be contacted by national and local authorities to control the quality of raw milk and commercially available milk products. Especially species-specific identification tools are of great interest for authenticity controls in animal-derived food products. Quantitative estimation, of course, of the adulteration level is also required.

Many different analytical methods have been developed for fraudulent adulteration detection of dairy products and

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animal-species identification. These methods are mainly based either on the detection of animal species-specific proteins or peptides, such as caseins and lactoglobulins, or species-specific DNA sequences [15]. This review reports the DNA-based analytical methods that have been developed for milk authenticity determination and detection of adulteration of milk-based products, as DNA is considered as the appropriate analyte for authenticity tests.

DNA-based techniques

DNA-based techniques have been successfully applied for milk authenticity testing. DNA is often chosen as the most appropriate analyte than proteins due to the higher stability of DNA comparing to proteins, especially during food processing [16], its conserved structure among all cells and the high sensitivity, specificity and reproducibility of DNA-based techniques [1, 17–19]. Polymerase chain reaction (PCR) is the first and essential step, until now, in DNA-based analytical methods, because the quantity of genomic DNA that is isolated from food samples is very low. Thus, animal species-specific DNA sequences are initially amplified by PCR. PCR-based milk authenticity methods are particularly focused on the analysis of mitochondrial DNA sequences, because its sequence is highly conservative within different animal species and gives high copy number in animal cells, increasing the specificity and the detectability of the method [20].

Several DNA-based techniques have been successfully applied to raw materials, heat-treated dairy products, matured cheeses, yogurts, butter and milk-based sweets, compared to protein-based techniques that could not always be applied and have to be chosen carefully depending on food matrix [6]. Quantitative determinations for food authentication have also been approached. DNA-based methods for milk authenticity testing are presented in Table 1 focusing on the detection limit of each method and the primers' sequences used for amplification of species-specific DNA sequences.

Restriction fragment length polymorphism analysis (RFLP)

Polymerase chain reaction (PCR) has been used for the first time by Plath et al. [21] for species identification of bovine milk in ovine or caprine milk and cheese samples obtained by European producers and local market. The identification was based on the amplification of β -casein gene using isolated genomic DNA. After restriction fragment length polymorphism analysis (RFLP) and polyacrylamide gel electrophoresis, the authors revealed that a different restriction site was present in ovine and caprine DNA, but is not

contained in bovine's DNA. The limit of detection of this method was 0.5% of cow's milk in ovine or caprine cheese. This method is restricted though, to the identification of the bovine species only [21].

The amplification of the conserved region of mitochondrial cytochrome b gene specific to mammals was used later for species identification (*Bos*, *Ovis*, *Capra*, *Bubalus*) in dairy products by PCR and restriction fragment length polymorphism analysis (PCR-RFLP) using agarose gel electrophoresis [22]. PCR-RFLP analysis was carried out again for discrimination between cattle and buffalo milk, while analysis of both genes encoding species-specific repeats (SSR) and cytochrome-b was used for detecting the fraudulent incorporation of cattle or buffalo milk in sheep's milk [23]. Home-made mozzarella cheese admixtures prepared by Otaviano et al., 2008 with different concentrations of bovine milk from the area of Brazil were tested by PCR-RFLP of β -casein gene. The amplicons were detected by agarose or polyacrylamide gel electrophoresis with ethidium bromide staining, while the detection limit was assessed at 1% of cow's milk in buffalo-made cheese [24].

In conclusion, PCR-RFLP analysis combined with gel electrophoresis showed high detection limit and very low high-throughput potential.

Agarose gel electrophoresis with intercalating dye staining

Agarose gel electrophoresis and visualization of the PCR products by DNA intercalating dyes were further used for detecting fraudulent substitution in milk-based products. The methods offer better detectability than PCR-RFLP, whereas their main disadvantage is that DNA electrophoresis provides information only about the size of the amplicons and does not give any evidence about the sequence of the products, whereas the lack of high-throughput and multiplicity potential. The efficiency of using different DNA target sequences was also studied.

Maudet et al. [25] showed that mitochondrial (mt) DNA may be more suitable than genomic DNA for species identification due to sequence variation and its higher number of DNA copies per cell, which could enable the more efficient amplification even of degraded DNA in low quantities. The method was based on the amplification of mtDNA control (D-loop) region using species-specific primers and subsequent analysis of PCR products with agarose gel electrophoresis and ethidium bromide staining. The method was applied first in home-made cheese samples with milk obtained by French local farmers and could detect less than 0.1% cow's milk in goat cheese. The method was then applied for the analysis of well-known French cheeses from local markets such as Roquefort, Bleu d' Auvergne, Comté, Beaufort, Camembert and Saint Marcellin [25]. De et al.

Table 1 DNA-based analytical methods for milk authentication

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Plath et al. [21]	<i>Universal primers for animal species, nuclear DNA</i> Forward TCCCTAATATC CAGTTGAGCC	<i>Universal primers for animal species, nuclear DNA</i> Reverse TCCTGGTACAGC AGAAAGGC	Guanidinium isothiocyanate/ EtOH/Silica paramagnetic particles/Gel filtration	PCR-restriction fragment length polymorphism analysis (RFLP)/ Polyacrylamide Gel electrophoresis	0.5% of cow species
Abdel-Rahman and Ahmed [23]	<i>Cattle- and buffalo-specific primers for gene encoding species-specific repeat (SSR)</i> Forward AAGCTTGTGACA GATAGAACGAT Reverse CAAGCTGTCTAG AATTCAGGGA <i>Sheep-specific primers SSR</i> Forward GTTAGGTGTAAT TAGCCTCGGAGAA Reverse AAGCATGACATT GCTGCTAAAGTTC	<i>Cattle- and buffalo-specific primers for cytochrome b gene</i> Forward CCATCCAACATC TCAGCATGATGAAA Reverse GCCCCTCAGAAT GATATTGTCTCTCA	Phenol/chloroform/ isoamyl alcohol extraction Ethanol precipitation	PCR-RFLP / Agarose gel electrophoresis	-
Ottaviano et al. [24]	<i>Universal primers for β-casein gene</i> Forward GATGAACCTCCAG GATAAAATC	<i>Universal primers for β-casein gene</i> Reverse ATAATAGGGAAG GGTCCCCG	Nonidet P40, SDS, Tris buffer, KCl/Ethanol precipitation	PCR-RFLP Gel electrophoresis	1% of cow species
Maudet et al. [25]	<i>Universal primers for mtDNA control gene (D-loop)</i> Forward ACATGAATTGGA GGACAACCCAGT Reverse CCTGAAAGTAAGA ACCAGATG	<i>Cow-specific primers</i> Forward TTCTATTAAAC TATTCCTTGAAC Reverse GATATACTATCA AGAATGAATTTGAC	DNeasy™ Tissue Kit (QIAGEN GmbH, Hilden, Germany)	PCR/Agarose gel electrophoresis	0.1% of cow species
De et al. [17]	<i>Common primer</i> Forward ACTAGATCACGA GCTTGATCACCATGC <i>Bovine-specific primer for mtDNA control gene (D-loop)</i> Reverse ATGCCTGGTAAA ATTCAATTAATAGCG	<i>Buffalo-specific primer for mtDNA control gene (D-loop)</i> Reverse GTTATGTGTGAG CATGGGCTGATTGGA	Phenol/Chloroform/ isopropanol Sodium acetate	PCR/Agarose Gel Electrophoresis	0.1% of cow and buffalo species
Bania et al. [26]	<i>Bovine-specific primers for cytochrome b gene</i> Forward GACCTCCAGCT CCATCAAACATCTCA TCT TGATGAA	<i>Bovine-specific primers for cytochrome b gene</i> Reverse CTAGAAAAAGTGT AAGACCCGTAATAT AAG	Phenol/chloroform/ isoamyl alcohol extraction Ethanol precipitation	PCR/Agarose Gel Electrophoresis	0.1% of cow species

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Rea et al. [27]	<i>Cow-specific primers for cytochrome b gene</i> Forward CGCATACGCAAT CTTACGATCCTCTTATT Reverse TGGAGGTGTGTA GTAGGGGGATT	<i>Buffalo-specific primers</i> Forward GCATACGCAATC TTACGATCCTCTTATT Reverse CATGGCTGAAT GGCCGGAACATCATA	-Phenol/chloroform/ isoamyl alcohol extraction Ethanol precipitation and -NaCl/EDTA/SDS/phenol/ chloroform extraction	PCR/Agarose gel electropho- resis	1% of cow species
Bottero et al. [29]	<i>Bovine-specific primers for 12S or 16S rRNA</i> Forward GTACTACTAGCA ACAGCTTA Reverse GCTTGATTCTCT TGGTGTAGAG <i>Caprine-specific primers</i> Forward CGCCCTCCAAAT CAATAAG Reverse AGTGTATCAGCT GCAGTAGGGTT	<i>Ovine-specific primers</i> Forward ATATCAACCACA CGAGAGGAGAC Reverse TAAACTGGAGAG TGGGAGAT	Dneasy Tissue Protocol (Qiagen GmbH, Hilden, Germany)	PCR/Agarose gel electropho- resis	0.5% of cow species
López-Calleja et al. [30]	<i>Universal primers for 12S RNA sequence</i> Forward GGTAATCTCGT GCCAGCCA Reverse TCCAGTATGCTT ACCTTGTACGAC	<i>Bovine-specific primers</i> Forward CTAGAGGAGCCT GTTCTATAATCGATAA Reverse TGGTTTCATAAT AACTTTCGCGCT	Wizard DNA cleanup kit (Promega, Madison, WI)	PCR/Agarose gel electropho- resis	0.1% of cow species
López-Calleja et al. [31]	<i>Cow-specific primers for 12S RNA sequence</i> Forward CTAGAGGAGCCT GTTCTATAATCGATAA Reverse AAATAGGGTTAG ATGCACTGAATCCAT	<i>Buffalo-specific primers</i> Forward CTAGAGGAGCCT GTTCTATAATCGATAA Reverse TTCATAATAAAT TTCGTGTGGGTGT	Wizard DNA cleanup kit (Promega, Madison, WI)	PCR/Agarose gel electropho- resis	0.1% of cow species
López-Calleja et al. [32]	<i>Goat-specific primers for 12S RNA sequence</i> Forward AACGTGTTAAAG CACTACATC Reverse GTCTTAGCTATA GTGTATCAGCTGCA	<i>Sheep-specific primers for 12S RNA sequence</i> Forward CTAGAGGAGCCT GTTCTATAATCGATAA Reverse GTCTCCCTCTCGT GTGGTTGAGATA	Wizard DNA cleanup kit (Promega, Madison, WI)	PCR/Agarose gel electropho- resis	0.1% of sheep species
Di Pinto et al. [38]	<i>Bovine-specific primers for mt cytochrome b gene</i> Forward GGCTTATATTAC GGGTCTTACACT	<i>Bovine-specific primers for mt cytochrome b gene</i> Reverse GGCAATTGCTAT GATGATAAAATGGA	DNeasy TM Isolation Kit (QIAGEN GmbH, Hilden, Germany)	PCR/Agarose gel electropho- resis	1.5% of cow species

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Felgini et al. [39]	<i>Bovine-specific primers for mt cytochrome oxidase subunit I gene</i> Forward GAACTCTGCTCG GAGACGAC <i>Common primer for cytochrome b gene</i> Forward GACCTCCAGCTCCA TCAAACATCTCATCT TGATGAAA <i>Cattle-specific Reverse primer</i> CTAGAAAAGTGTAAAG ACCCGTAATATAAG	<i>Bovine-specific primers for mt cytochrome oxidase subunit I gene</i> Reverse AGCACCAATTAT TAGGGGAAC <i>Goat-specific Reverse primer</i> CTCGACAAATGTGAG TTACAGAGGGA <i>Sheep-specific Reverse primer</i> CTATGAATGCTGTGGCTA TTGTCGCA	Nucleospin Food kit purification (Macherey–Nagel, Düren, Germany)	PCR/Agarose gel electrophoresis	0.5% of cow species
Mašková et al. [28]	<i>Forward</i> GACCTCCAGCTCCA TCAAACATCTCATCT TGATGAAA <i>Cattle-specific Reverse primer</i> CTAGAAAAGTGTAAAG ACCCGTAATATAAG	<i>Goat-specific Reverse primer</i> CTCGACAAATGTGAG TTACAGAGGGA <i>Sheep-specific Reverse primer</i> CTATGAATGCTGTGGCTA TTGTCGCA	DNA isolation kit Invisorb Spin Food I (Invitex, Co., Berlin, Germany)	PCR/Agarose gel electrophoresis	1% of cow species
Kotowicz et al. [40]	<i>Bovine-specific primers for mtDNA control gene (D-loop)</i> Forward CAATAACTCAAC ACAGAATTTC Reverse CGTGATCTAATG GTAAGGAATA <i>Goat-specific primers for 12S rRNA gene</i> Forward AAACGTGTTAAA GCACTACATC Reverse GTCTTAGCTATA GTGTATCAGCTGCA <i>Bovine-specific for 12S rRNA sequence</i> Forward GTACTACTAGCA ACAGCTTA Reverse GCTTGATTTCTT TGGTGTAGAG	<i>Goat-specific primers</i> Forward CCAACATGCGTA TCCCGT Reverse AGCGGATGCATG ATGAAATG <i>Sheep-specific primers</i> Forward CTAGAGGAGCCT GTTCTATAATCGATAA Reverse GTCTCCTCTCGT GTGGTTGAGATA <i>Goat-specific primers</i> Forward CGCCCTCCAAAT CAATAAG Reverse AGTGTATCAGCT GCAGTAGGGTT	Phenol/chloroform/ extraction Ethanol precipitation	PCR/Agarose gel electrophoresis	1% of cow species
Diaz et al. [33]	<i>Goat-specific primers for 12S rRNA gene</i> Forward AAACGTGTTAAA GCACTACATC Reverse GTCTTAGCTATA GTGTATCAGCTGCA <i>Bovine-specific for 12S rRNA sequence</i> Forward GTACTACTAGCA ACAGCTTA Reverse GCTTGATTTCTT TGGTGTAGAG	<i>Goat-specific primers</i> Forward CCAACATGCGTA TCCCGT Reverse AGCGGATGCATG ATGAAATG	Wizard DNA cleanup kit (Promega, Madison, WI)	PCR/Agarose gel electrophoresis	1% of goat species
Mafta et al. [34]	<i>Bovine-specific for 12S rRNA sequence</i> Forward GTACTACTAGCA ACAGCTTA Reverse GCTTGATTTCTT TGGTGTAGAG	<i>Goat-specific primers</i> Forward CGCCCTCCAAAT CAATAAG Reverse AGTGTATCAGCT GCAGTAGGGTT	Guanidinium isothiocyanate/ EtOH Silica paramagnetic particles	PCR/Agarose gel electrophoresis	0.1% of cow species
Rodrigues et al. [35]	<i>Bovine-specific primers for mtDNA</i> Forward CAATAACTCAAC ACAGAATTTC Reverse CGTGATCTAATG GTAAGGAATA	<i>Goat-specific primers</i> Forward CCAACATGCGTA TCCCGT Reverse AGCGGATGCATG ATGAAATG	Phenol/chloroform/isoamyl alcohol extraction Ethanol precipitation	PCR/Agarose gel electrophoresis	0.5% of cow species

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Golinelli et al. [36]	<i>Bovine-specific primers for 12S RNA sequence</i> Forward GTACTACTAGCA ACAGCTTA Reverse GCTTGATTCTCT TGGTGTAGAG <i>Common primers for ND1 gene</i> Forward GAAAAGGTCCAA ATGTCGTAGGT Reverse TCCGATTAGTGC GTATTTTGAGT	<i>Goat-specific primers</i> Forward CGCCCTCCTCAAAT CAATAAG Reverse AGTGTATCAGCT GCAGTAGGGTT <i>Bovine-specific primer</i> Forward CTCATATTAT CCTAGCACCTATCA	DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany)	PCR/Agarose gel electrophoresis	0.5% of cow species
Bai et al. [41]			Wizard DNA cleanup kit (Promega, Madison, WI)	PCR/Agarose gel electrophoresis	0.1% of cow species
Tortorici et al. [37]	<i>Bovine-specific primers for 12S RNA sequence</i> Forward GTACTACTAGCA ACAGCTTA Reverse GCTTGATTCTCT TGGTGTAGAG <i>Goat-specific primers</i> Forward GCCCTCCAAATC AATAAG Reverse AGTGTATCAGCT GCAGTAGGGTT	<i>Ewe-specific primers</i> Forward TATCAACCACAC GAGAGGAGAC Reverse TAAACTGGAGAG TGGGAGAT <i>Universal primers for k casein gene</i> Reverse AGGGATTCTGT TTATCCTGAT	Hexadecyltrimethylammonium bromide (CTAB) method	PCR/Agarose gel electrophoresis	0.1% of cow, ewe and goat species
Reale et al. [42]			DNeasy TM Tissue Kit (Qiagen) with some modifications	PCR/Single nucleotide primer extension/Capillary electrophoresis	0.1% of cow species

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Gonçalves et al. [43]	<i>Cow-specific primers for mt DNA</i> Forward GCCGGGCACAATC GAAAACAAAT Reverse CTTCAGCTTTGG GGGTGATG and Forward GTTAACAGC TAAACACCCTAGCT Reverse AGGTTTGACTCC TCTTTTACCA <i>Buffalo-specific primers</i> Forward CCAAAATTAAAC ACAAATCCCGCAA Reverse CATGGTCGTGG TTGAATTCCA and Forward GAATTTATC TCAATTAGTAACGCAAC Reverse CTACTAATGTGA GGAATGCCACT <i>Caprine-specific primers</i> Forward CACCAAAATTCA ACACAATACCACAT Reverse AGCGTTATCTTT GTAATAGGTTTTGT	<i>Caprine-specific primers</i> and Forward CTACCACAA CCCAGAAATTAAACAG Reverse TAAGGGTAACAA GGGGGAGG <i>Ovine-specific primers</i> Forward CCAAAATTCAAC ACAATACCACAC Reverse TTCCATGTGAGA ATGATGATGACA and Forward CGTAGATGT AGTATGACTTTTCCT Reverse GTGAAGTTAGTT AGGAGAGTAATTATA <i>Common primers</i> Forward TCCCAGTACGAA AGGACAAAGA Reverse CAATTACCGGGC TCTGCCA	Standard phenol – chloroform extraction method	Quadruplex PCR and capillary electrophoresis	1% of cow, buffalo, goat and sheep species
López-Calleja et al. [45]	<i>Goat-specific primers for 12S rRNA gene</i> Forward AAAGGACTTGGC GGTGCTT Reverse TGACCTAACGTC TTTATGTGTGTG <i>Common Taqman probe</i> 6FAM-AGAGGAGCCTGT TCTATAATCGATAAA CCCCG-TAMRA <i>Bovine-specific primers for cytochrome b gene</i> Forward CGGAGTAACTCT TCTGCTCACAGT Reverse GGATTGCTGATA AGAGGTGTGTG	<i>Mammalian-specific primers</i> Same Forward AAAGGACTT GGCGGTGCTT Reverse GGGTTTGCTGAA GATGGCG	Wizard DNA Clean-up system (Promega, Madison, WI, USA)	Real-time PCR Taqman probes	0.6% of goat species
Zhang et al. [46]		Taqman Probe 6FAM-TGAGGACAA ATATCATCATTTCTGA GGAGCW ARGTYA- TAMRA	CTAB method for cheese/ Promega Wizard Magnetic kit (Promega, Madison, WI, USA) for milk	Real-time PCR Fluorescent probe	35 pg of bovine DNA

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Lopparelli et al. [49]	<i>Cow and buffalo-specific primers for cytochrome b gene</i> Forward TTGGGCCCCCTGC AGTTC Reverse GGTCGAGGTGC CAACAC Common Taqman probe AGC AGAGTCTTCACCAAC	<i>Cow-specific primers for cytochrome b gene</i> Forward CAAGAACACTAA TGACTAACATTCGAAAG Reverse AAATGTTTGATG GGGCTGGA Taqman probe CCACCCACT AATAAA	Invisorb Cell Mini Kit (Invitex) for milk Invisorb Tissue Mini Kit (Invitex) for cheese	Real-time PCR Taqman probes	0.1% of cow species
Dalmaso et al. [48]	<i>Cow and buffalo-specific primers for cytochrome b gene</i> Forward CAAACGGAGCTT CAATGTTTT Reverse CGTATCCTATAA ATGCTGTGGCTATTACT	<i>Taqman probe specific to cow</i> 6FAM-TTATATATGCAC GTAGGACG-MGB <i>Taqman probe specific to buffalo</i> VIC-TTATATATACACGTA GGACGAG-MGB	Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany)	Real-time PCR Taqman probes	2% of cow species
Cottenet et al. [47]	<i>Cow and buffalo-specific primers for cytochrome b gene</i> Forward AATACACTACAC ATCCGACACACAA Reverse GCTCCGTTGCG TGTATGTATC	<i>Fluorescent probe specific to cow</i> 6FAM-CTCTGTTACCCA TATCTG <i>Fluorescent probe specific to buffalo</i> VIC-CTCCGTCGCCACACA	CTAB method QIAquick column (Qiagen) purification	Real-time PCR Fluorescent probes	1% of cow species
Drummond et al. [50]	<i>Bovine-specific primers for cytochrome b gene</i> Forward CGGAGTAATCCT TCTGCTCACAGT Reverse GGATTGCTGATA AGAGGTTGGTG Taqman probe FAM-CAT GAGGACAAATATC-MGB	<i>Buffalo-specific primers for 16S rRNA gene</i> Forward TCAGCCCCAAAGA AAAATAAACCA Reverse GTCACCCCAACC GAAACTGT Taqman probe FAM-TAA GGARTAACAAACAMTCT-MGB	Guanidinium isothiocyanate/ EtOH Silica particles	Duplex Real-time PCR / Taqman probes	1% of cow species

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Rentsch et al. [13]	<i>Bovine-specific primers for nuclear DNA</i> Forward AGTTAGAGATTG AGAGCCATATCTCTCC Reverse TTGATAAGATCA TTGTAGTCATGTTG Taqman probe FAM-TGG TGACATGCCGCAACT AGACACG-BHQ1 <i>Goat-specific primers</i> Forward CACTTATCCTC CCATTCATCATCAC Reverse TCTTTAATGGTG TAGTAAAGGGTGAAATG Taqman probe JOE-CCTCGC CATAGTCCACCTGCTCT TCC-BHQ1	<i>Sheep-specific primers</i> Forward TTTCGCCTTTTCA CTTTATTTTCCC Reverse GAAATCCTGTGG GGTTGTTGG Taqman probe ROX-CGC AGCCCTCGCCATAGT TCACCT-BHQ2 <i>Buffalo-specific primers</i> Forward TCTGGGGAGGAT TCTCAGTAG Reverse AAGTGCTGGGAT AATGAATGG Taqman probe Cy5-AGC AACCCTCACCCGATTCT TCGC-BHQ2	Guanidinium isothiocyanate Wizard Plus Miniprep DNA purification system (Promega, Madison, USA)	Quadruplex Real-time PCR / Taqman probes	0.2% of cow species
Domenico et al. [51]	<i>Bovine-specific primers for cytochrome b gene</i> Forward GAACTACGGGCTG AATCATCCGA Reverse GGTAGGACGTAT CCTATAAATGCTGTG Taqman probe 6FAM-TAA TCCTTCTGCTCACAG TAAT- MGBNFQ <i>Goat-specific primers for 12S rRNA gene</i> Forward AGGTCAAGGTGT AAGCCATGGAA Reverse CTAAATCCTCCT TTGGTCATTAATTCA Taqman probe 6FAM-TTA AGAAAAATTAAATACGAA AGCC-MGBNFQ	<i>Sheep-specific primers for cytochrome b gene</i> Forward ACGAGGCCTATA CTATGGATCATATACC Reverse TCCTCATGGTAA AACATAGCCTATGAATG Taqman probe 6FAM-CTC CTATTTGCGACAATA G-MGBNFQ <i>Buffalo-specific primers for 12S rRNA gene</i> Forward GTAACTATGAA ATGGGAAGAAATGG Reverse TTAAGTCTAAAT CCTCCTTTGGTTATTAAT Taqman probe 6FAM-ACA CCAAGAACACCCCAAC -MGBNFQ	Maxwell 16 Tissue DNA Purification kit (Promega, Madison, WI)	Real-time PCR Taqman probes	< 1% of cow, goat, sheep and buffalo species

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Ganopoulos et al. [16]	<i>Bovine-specific primers for D-loop mitochondrial gene</i>	<i>Ovine-specific primers</i>	Nucleospin Tissue kit (Macherey–Nagel, Duren, Germany)	Real-time PCR HRM analysis	0.1% of cow species 1% of caprine and ovine species
	Forward AACCAATATTA	Forward ACACAACCTTCTA			
	Reverse AACCACTAGCT	Reverse AAACAATGAGGG			
	Reverse AACCACTAGCT	Reverse AACCACTAGCT			
	<i>Caprine-specific primers</i>				
Sakaridis et al. [52]	Forward CCCAATCCTAAC		Nucleospin Food kit (Macherey–Nagel, Duren, Germany)	Real-time PCR HRM analysis	1% of cow species
	Reverse TGTGTAGGCGAG				
	CGGTGTAA				
	<i>Bovine-specific primers for D-loop gene</i>	<i>Buffalo-specific primers for 12S rRNA sequence</i>			
	Forward AACCAATATTA	Forward CTAGAGGAGCCT			
Agrimonti et al. [53]	Reverse AAATAGGGTTAG	Reverse TTCATAATAACT	Hexadecyltrimethylammonium bromide (CTAB) method Ethanol precipitation	Quadruplex real-time PCR HRM analysis	0.1% of cow species
	ATGCACTGAATCCAT	TTCGTGTGGGTGT			
	<i>Bovine-specific primers for 12S rRNA gene</i>	<i>Buffalo-specific primers for cytochrome b gene</i>			
	Forward GCTAACATAACA	Forward CCTAGTTCTCTC			
	Reverse TTGCGTAGGTAA	Reverse TAGGCATTGGCT			
Agrimonti et al. [53]	Reverse AACCACTAGCT	Reverse AACCACTAGCT	Hexadecyltrimethylammonium bromide (CTAB) method Ethanol precipitation	Quadruplex real-time PCR HRM analysis	0.1% of cow species
	Reverse AACCACTAGCT	Reverse AACCACTAGCT			
	Reverse AACCACTAGCT	Reverse AACCACTAGCT			
	Reverse AACCACTAGCT	Reverse AACCACTAGCT			
	Reverse AACCACTAGCT	Reverse AACCACTAGCT			

Table 1 (continued)

References	Primers and probes SEQUENCE (5' → 3')	Primers and probes sequence (5' → 3')	DNA extraction method	Method	Detection limit
Liao et al. [44]	<i>Bovine-specific for cytochrome b gene</i> Forward CGACCTTCCAGC CCCATCGA Reverse AGCCGTAGTTCA CGTCTCGGC <i>Bovine-specific for 12S rRNA gene</i> Forward CTAGAGGAGCCT GTTCTATAATCGATAA Reverse AAATAGGGTTAG ATGCACTGAATCCAT	<i>Goat-specific for ATP6 gene</i> Forward TATTAGGCCTCC CCCTTGTT Reverse CCCTGCTCATAA GGGAATAGCCCC	Tris-phenol Phenol/chloroform/ isoamyl alcohol Ethanol precipitation	Real-time PCR Intercalating Fluorescent Dye	0.1% of cow species
Kounelli et al. [54]	<i>Bovine-specific primers for cytochrome b gene</i> Forward AGTTAGAGATTG AGAGCCATATACTCTCC Reverse Biotin-TTGATAAGA TCATTGTCAGTCATG TTG Probe TGGTGACATGCC GCAACTAGACACG <i>Sheep-specific primers for cytochrome b gene</i> Forward Biotin-CCAACA TGCCTTTAAACCCCTCAA Reverse GGAACGTAGCC TTCTGACTCG Probe TGGCTTTTCCTTCCTCC GCCAGTCTC	<i>Goat-specific primers for 12S rRNA gene</i> Forward CACTTTATCCTC CCATTCATCATCAC Reverse Biotin-TCTTTAATG GTGTAGTAAGGG TGA AATG Probe CCTCGCCATAGT CCACCTGCTCTTCC	NucleoSpin Food kit (Macherey–Nagel, Düren, Germany)	PCR/Hybridization assay on fluorescent microspheres	0.01% of cow species in goat's milk 0.05% of cow species in sheep's milk
Beltramo et al. [55]	Species-specific primers to cytochrome b oxidase gene	Primers supplied with Meat LCD Array kit (Chipron)	NucleoSpin Food kit (Macherey–Nagel, Düren, Germany)	PCR/Hybridization assay on a biochip	0.1% of cattle, goat and buffalo-species

[17] also exploited the mtDNA D-loop region of the development of simplex and duplex PCR for the species-specific identification of cattle and buffalo milk in milk (raw and heated) and cheese samples obtained by the National Dairy Research Institute in India. The detection limit was 0.1% [17].

Bania et al. [26] had used species-specific primers targeting the cytochrome b gene for the detection of the adulteration of goat with cow's milk. The detection was based on agarose gel electrophoresis and ethidium bromide staining and because of the use of specific primers did not need the extra step of restriction enzyme analysis. As low as 0.1% of cow's milk (from different commercial sources) in goat's milk (from local French farmers) was detected by this method [26]. Rea et al., 2001 had also exploited the sequence of cytochrome b gene in mitochondrion for the development of a duplex PCR with subsequent agarose gel analysis for the detection of bovine in water buffalo milk and mozzarella home-made cheese with a sensitivity limit of 1%. Non-marketed milk samples were obtained by a Japanese market and an Italian cheese factory [27]. Similarly, the amplification of the conserved region of the same mitochondrial gene was used again later for the detection of 1% cow milk in goat and sheep-based cheeses by PCR and agarose gel electrophoresis with ethidium bromide staining. The cheese samples were prepared by 22 different producers from five European countries and purchased from Czech and Italian stores (Balkan cheeses, Greek Feta and cheeses from Italy, France and The Netherlands) [28].

The amplification of conserved regions of mitochondrial 12S and 16S rRNA genes among different animal species was also tested for the detection of milk adulteration with cow milk [29–37]. Bottero et al. [29] developed a triplex PCR for identification of cow, goat and sheep milk in cheese samples (purchased from Italian markets) based on agarose gel electrophoresis with ethidium bromide staining. The sensitivity of the method was 0.5% of bovine in caprine milk. The authors used also PCR-RFLP analysis for the confirmation of the results [29]. López-Calleja et al. reported (a) a method for the detection of cow milk in sheep's and goat's milk, (b) a method for the detection of goat milk in sheep's milk and (c) a method for the detection of cow milk in water buffalo milk and mozzarella cheese. Mozzarella cheese is a typical Italian product marketed worldwide that is exclusively made from water buffalo (*Bubalus bubalus*) milk. Milk samples were collected from a local Spanish farm and local markets. First, the authors amplified a conserved region of 12S rRNA sequence using universal primers within different animal species and after sequencing of the PCR products for cow, sheep and goat species, they designed cow-specific primers. The methods were based on agarose gel electrophoresis and staining with ethidium bromide and enabled the specific detection of cow's DNA in lab-prepared binary

mixtures of milk and cheese with a sensitivity threshold of 0.1% [30–32]. Díaz et al. [33] reported a similar PCR-based method to detect as low as 1% of caprine milk in ovine cheese purchased by a local industry and different markets in Spain (Majorero goat's milk cheese and Torta del Casar sheep's milk cheese) [33]. Mafra et al. [34], Rodrigues et al. [35] and Golinelli et al. [36] settled a duplex PCR with subsequent detection in agarose gel with ethidium bromide or GelRed staining for the detection of cow milk in goat milk and cheese. The method was validated with prepared binary mixtures of cheese. The detection limit of the three methods was 0.1% cow's milk in home-made goat's cheese, 0.5% cow's milk in goat's milk and 0.5% of cow's milk in goat's cheese, respectively. Milk and cheese samples were obtained by local farmers, industries and markets [34–36]. Moreover, Tortorici et al. [37] developed a duplex PCR for the amplification of cow, ewe and goat DNA sequences specific to 12S rRNA gene. The amplicons were detected again by electrophoresis. As low as 0.1% of animal species were detected in binary milk samples from local Italian farms and home-made binary cheese samples by the proposed method [37].

Mozzarella cheese was also tested for substitution of buffalo with bovine milk by species identification of bovine-specific mitochondrial cytochrome b gene and mitochondrial cytochrome oxidase subunit I gene. The detection limit was 1.5 and 0.5%, respectively, of cow milk in prepared cheese samples [38, 39]. Moreover, Kotowicz et al. [40] developed a duplex PCR with agarose gel analysis of the PCR products for detection of 1% bovine in goat milk using primers specific to bovine species. The milk samples were obtained by Polish farmers and market [40]. Bai et al. [41], finally, exploited the amplification of mitochondrial *ND1* gene by means of PCR and agarose gel electrophoresis for the detection of 0.1% bovine in yak milk in prepared binary raw and heat-treated milk samples from a local Chinese farm [41].

Capillary electrophoresis

Capillary electrophoresis (CE) was then introduced for the rapid detection of milk adulteration, significantly lowering the time of the analysis. A new method based on species-specific single nucleotide polymorphism (SNP) detection was settled by Reale et al. [42] for milk substitution determination with bovine milk. The method includes amplification reaction of the conserved region of k-casein gene between four different animal species using the same pair of primers for amplification and subsequent allele discrimination of three SNPs, after cleaning of the PCR products from excess of primers and dNTPs through exonuclease I and shrimp alkaline phosphatase (Exo-SAP) treatment, with minisequencing (single nucleotide) primer extension reaction using fluorescently labeled dideoxynucleotides (ddNTPs) and the ABI Prism SNaPshot™ Multiplex System. The

primer extension products were again purified with Exo-SAP. The kind of nucleotide added during the reaction was determined by capillary electrophoresis using Hi-Dye formamide (Fig. 1). The authors could detect simultaneously four animal species (cattle, goat, sheep and water buffalo) in raw and pasteurized milk and mozzarella and caciotta cheese products obtained by local Italian farmers, as well as to detect as low as 0.1% proportion of adulteration of bovine in buffalo milk by serial dilutions of isolated DNA of the two species [42].

A quadruplex PCR reaction with subsequent capillary electrophoresis using fluorescently labeled primers with 6-carboxyfluorescein has been developed by Gonçalves et al. [43] for the simultaneous detection of four animal species (buffalo, cow, sheep and goat) based on the fragment size comparison of the newly synthesized PCR products. The CE separation was completed within 700 s. The method targeted the mitochondrial-specific DNA sequences in commercial dairy products by local Portuguese market: milk, powder milk, cheese, cottage cheese, butter, cream and yogurt. A conserved region between the four species was also amplified by a common pair of primers serving as an internal positive control of the reaction, thereby balancing the potential fluctuations during the analysis. The detection limit of adulteration in binary home-made milk mixtures of the described method was 1% [43].

Advanced methods for tracing milk adulteration

Real-time PCR

Real-time PCR has subsequently been introduced in milk authenticity analysis aiming to increase the multiplicity and the high-throughput of the method, reducing also the time of the analysis but with comparable LODs. Real-time PCR is commonly used in routine analysis with numerous applications. The advantages of real-time PCR are: (a) there is no need for post-PCR detection and identification of the PCR products, reducing, therefore, the time of the analysis and (b) using different fluorophores, up to six different DNA sequences can be simultaneously analyzed. Both DNA-intercalating dyes and specific Taqman and simply fluorescent

species-specific oligonucleotide probes have been used as detection systems in real-time PCR for milk authenticity testing. Quantitative real-time PCR has also been developed. Real-time PCR has two main detection approaches: (a) using DNA intercalating fluorescent dyes for the detection of the newly synthesized double-stranded DNA sequences and (b) using specific fluorescent DNA probes for the detection and identification of the amplicons.

Real-time PCR with fluorescent DNA-intercalating dyes

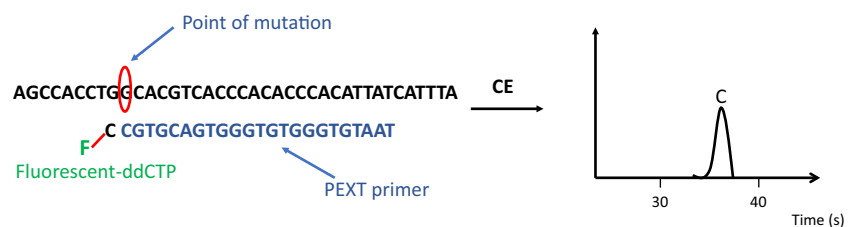
Liao et al. [44] presented a novel DNA extraction method for qualitative and quantitative determination of milk powder purchased by a local Chinese market. Milk powder samples were first washed to separate milk fats and impurities. DNA was then isolated using a usual extraction method with an extra step of tris-phenol addition. The isolated DNA was evaluated by UV absorption, while its integrity was confirmed by agarose gel electrophoresis. Both conventional and real-time PCR were then carried out to detect adulteration of goat milk powder with cow milk powder. The amplification was based on mitochondrial cytochrome b gene. The amplicons were detected by agarose gel electrophoresis for conventional PCR and with fluorescent dye SYBR Green for real-time PCR. The detection limit of conventional PCR was 0.1%, while the quantification limit of real-time PCR was found to be 0.5% with good accuracy [44].

Real-time PCR with Taqman or fluorescent probes

López-Calleja et al. [45] settled a method for quantification of goat's milk in sheep's milk based on real-time PCR of goat species using goat-specific primers for 12S rRNA gene. A parallel amplification of mammalian-specific sequence was also conducted serving as an internal standard control. The identification of the amplicons was accomplished by a common goat- and mammalian-specific Taqman probe. The method's sensitivity was 0.6% of goat milk in raw and heat-treated binary milk mixtures. Samples were collected by a local Spanish farm and several local markets [45].

Zhang et al. [46] used real-time PCR and Taqman-specific probe for the detection of cow's milk in milk and cheese samples (obtained by local UK retailers and

Fig. 1 Minisequencing primer extension reaction using fluorescently labeled ddNTPs and capillary electrophoresis for the detection of animal species-specific single-nucleotide polymorphisms. CE capillary electrophoresis



producers) based on the amplification of bovine-specific DNA sequence of cytochrome b gene. The detection limit of the method was 35 pg of bovine DNA that was determined by a calibration curve with serial dilutions of bovine DNA [46].

The amplification of the conserved region of mitochondrial cytochrome b was also exploited for simultaneous amplification of DNA derived from cow and buffalo milk or cheese from Asian milk collection centers located in China, India and Pakistan or local markets or cheese manufacturers in Italy. The identification was based on species-specific Taqman or fluorescent probes, whereas the authors were able to detect down to 1 or 2% of cow milk in buffalo milk, respectively and 0.1% of cow milk in buffalo home-made and commercial mozzarella cheese obtained by a local Italian farm and local markets [47–49]. The last authors compared their results with SYBR Green I-based real-time PCR. A method was also settled by Drummond et al. [50] by means of real-time PCR to quantify bovine contamination in buffalo commercial products from local Brazil market using Taqman-specific probes or SYBR Green I. As low as 1% of bovine cheese in binary cheese mixtures was detected by this method [50].

A quadruplex real-time PCR using Taqman probes was developed by Rentsch et al. [13] for simultaneous determination of cow, sheep, goat and buffalo milk in milk, cheese and yogurt samples from local Swiss suppliers, as well as in home-made French cheese samples. The sensitivity of the method was 0.2% of cow milk [13]. A quantitative method based on competitive real-time PCR using Taqman probes and an internal amplification control was set by Domenico et al. [51] for animal-species identification and fraud detection of dairy products. Milk samples were directly obtained by local Italian farms, while commercial cheeses from local retailers were also analyzed: Parmigiano, caciotta, pecorino, buffalo mozzarella and goat fresh and ripened cheese. The species analyzed were bovine, buffalo, ovine and caprine and was based on the amplification of DNA sequences specific to mitochondrial 12S rRNA and cytochrome b gene, providing with a detection limit lower than 1% that was determined by serial dilution of isolated DNA from all species. The internal controls used in this study were constructed and designed as a nontarget chimeric DNA sequence containing a portion of the acetyl-coenzyme A carboxylase gene (ACC) and a portion of cytB or 12S rRNA mtDNA sequence complementary to the primers. In each reaction tube, the DNA internal control was co-amplified with the target DNA using the same primer pair to balance variances during DNA isolation procedure and the amplification reaction. In that way, the more accurate determination of the initial DNA amount present in the sample was accomplished [51].

Real-time PCR and high-resolution melting analysis (HRM)

Ganopoulos et al. [16] presented a method that included amplification of bovine species by real-time PCR with fluorescent Syto9 dye and subsequent high-resolution melting analysis (HRM) for confirmation of the synthesis of the desired amplified products providing a detection limit of 0.1%. In parallel, they have set a duplex real-time PCR and HRM analysis for goat and sheep species identification in Greek Feta cheese (home-made or obtained by local markets or manufacturers) with a limit of detection of 1% [16]. Sakaridis et al. [53] were also used the same techniques exploiting short DNA sequences (barcodes, Bar-HRM) for quantitative detection of bovine milk in buffalo dairy products (containing mozzarella di bufala campana, burro di bufala, buffalo butter, buffalo cream, buffalo yoghurt, buffalo rice pudding, kasan di pi, a traditional sweet made entirely from buffalo milk, buffalo milk and buffalo ariani yoghurt) from Greek markets achieving a quantitation limit of 1% [52].

A quadruplex (cow, goat, sheep and buffalo) real-time PCR based on DNA intercalating fluorescent dye (SYBR GreenER) and HRM analysis has been also developed by Agrimonti et al. [54] offering a detection limit of 0.1% for cow species in home-made cheese mixtures. Their results from the analysis of commercially available Italian dairy products, such as mozzarella, ricotta, scamorza, parmesan and feta cheese, cream and yogurt samples, indicated the applicability of the method [53].

Hybridization assays

Hybridization assays on different solid supports have been developed for milk authentication, providing the advantages of a rapid, specific and sensitive species detection and contemporaneous identification along with the increased number of the different animal-specific DNA sequences that can be simultaneously analyzed.

Kounelli et al. [55] developed a hybridization assay on spectrally distinct fluorescent microspheres for milk authentication. The authors achieved a lower detection limit of 0.01% of cow species in goat's yogurt and 0.05% in sheep's yogurt by this method. Yogurt samples were purchased by Greek local markets. The method included (a) DNA extraction from mono-species or binary mixtures of yogurt samples, (b) PCR amplification using primers specific for each target and (c) identification of the amplicons by hybridization assay on spectrally encoded fluorescent microspheres that are coupled with animal species-specific DNA probes. These microspheres contain two fluorescent dyes in different ratios producing up to 100 distinct groups. The amplicons were biotinylated at one end, while the hybrids on the microspheres were detected by streptavidin–phycoerythrin

(fluorophore) conjugate (Fig. 2). The microspheres were finally analyzed one by one with a flow cytometer. Apart from the detection limit, this method gives a multiplexing potential and the possibility for simultaneous analysis of different analytes such as proteins, nucleic acids and small molecules (e.g., antibiotics) [54].

A biochip was developed by Beltramo et al. [56] for cattle, goat and buffalo species identification in milk admixtures with a sensitivity level of 0.1%, increasing the multiplicity of the analysis. All samples were from local Italian markets. The principle of the method is presented in Fig. 3. Biotinylated amplicons specific to cytochrome b oxidase gene of all three species are hybridized to specific probes that are immobilized onto the biochip surface (LCD Array kit for species identification on food, Chipron). The hybrids were detected by streptavidin-peroxide (HRP) conjugate, HRP chromogenic substrate and a LCD Array scanner. With

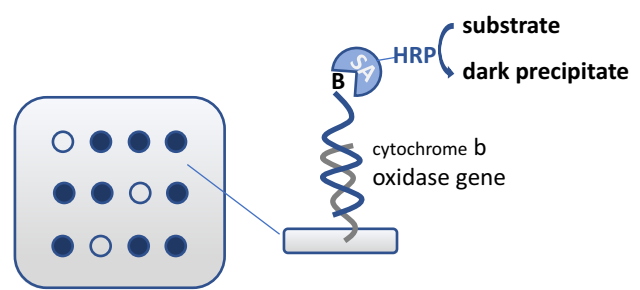


Fig. 3 A biochip for animal species identification. The hybrids formed between biotinylated amplicons and species-specific probes immobilized on the biochip were detected by streptavidin-peroxide (HRP) conjugate, HRP chromogenic substrate and a LCD array scanner. With this biochip, 25 different animal species could be simultaneously detected

this biochip, 25 different animal species could be detected simultaneously [55].

Future perspectives

Other advanced DNA-based methods widely used in food authentication has to be also applied for milk authenticity controls. DNA barcoding, next-generation sequence (NGS), DNA microarrays, as well as isothermal amplification and digital PCR of species-specific DNA sequences are well-established techniques that have been applied, alone or in combination, for species identification and food adulteration detection. Thus, it is worth to be further applied for the detection of milk adulteration in dairy products. The validation of the experimental performance of these techniques remains still a challenge, especially when a complex matrix, such as food, is used [56].

DNA barcoding

In the last decades, the identification of short DNA sequences, called DNA barcodes, have been widely exploited for species discrimination. DNA barcodes are small fragments and usually species-specific. The most common DNA fragments used are located at the mitochondrial region of cytochrome c oxidase (COI) for animal species or at the RuBisCo large subunit (*rbcL*) and maturase K (*matK*) genes for plants [56, 57]. Applications include discrimination between plant species such as hippophae, saffron and other herbs [58–61], as well as species identification in fish-samples [62, 63]. The experimental part involves amplification of these short DNA sequence and evaluation with reference barcodes. The use of DNA barcoding as an identification tool has proven to be very useful for the detection of the accidental or intentional substitutions of animal or plant species, as mislabeling and adulteration has found to be widespread among various processed food products.

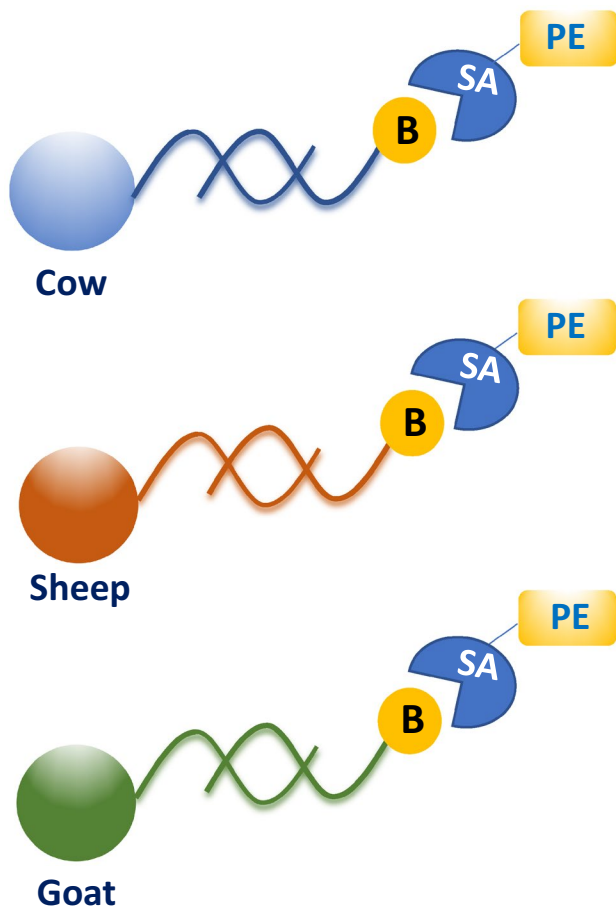


Fig. 2 Simultaneous identification of animal-specific DNA sequences using different fluorescent microspheres coupled to species-specific probes. The biotinylated (B) hybrids were detected by a streptavidin-phycoerythrin (SA-PE) molecule, where PE served as a reporter fluorophore, and flow cytometric analysis. Up to 100 different DNA sequences can be detected by this method

Well-defined reference libraries have been developed to enable DNA barcoding application. Barcode of Life Data Systems, commonly known as BOLD, is widely used by researchers, as it is a sequence database specifically devoted to DNA barcoding. It also provides an online platform for analyzing DNA sequences. However, many criticisms due to specific limitations, such as the universality of the method, have been faced by this technique [64, 65].

Next-generation sequence (NGS) and DNA microarrays

Next-generation sequencing and DNA microarrays (chips) have also emerged as promising tools in authenticating issues. Both techniques, along with DNA barcoding, utilize short DNA sequences (molecular markers) to identify an organism. DNA microarrays are constructed by the immobilization or direct synthesis of DNA detection probes on the surface of a solid support, usually a glass or plastic chip. Fluorescence or chemiluminescence is used to detect the hybrids formed on the surface of the chip. Microarrays allow the simultaneous detection of hundreds or thousands of different DNA sequences in a sample, reducing the analysis time and the sample volume, while they have increased dramatically the high-throughput of DNA-based methods with various applications [65]. However, their application in food authentication is poorly documented.

During last years, there has been a significant progress in DNA sequencing technologies. Next generation sequencing systems provide fast and simultaneous sequencing of hundreds or thousands of different molecules. Thus, a large number of sequencing data are collected rapidly. With this technology, the DNA target is fragmented and immobilized on a solid support, where the fragments are amplified and sequenced [65]. This technique has limited number of reports in food authenticity testing. It has been applied for the discrimination of herbs and plants and identification of mammal, meat and fish species [66–70]. The main disadvantage of these techniques still remains the high cost of the analysis and the complex workflow [66]. Although NGS is a very powerful tool for species identification, it may lead to uncertain results in the presence of multiple different species-specific DNA sequences in the same sample. A combination of DNA barcoding with microarrays or NGS is now the guideline for most researchers.

Isothermal amplification

Isothermal amplification technologies have dealt with some of the limitations of conventional PCR such as PCR inhibitors, as they do not depend on complex thermal cycling parameters and only one step at constant temperature is needed. During last years, many types of isothermal

amplification strategies have been developed exhibiting higher amplification efficiency than simple PCR. They have also provided us with reduced amplification time, simplicity, no special equipment is required, while their capability in portability and integration onto microfluidic devices has earned the interest of researchers for their application into the food safety area, such as animal species identification in plants, herbs and meat food samples [66, 71–75].

Digital PCR

Digital PCR facilitates the real-time PCR technology, as no reference calibration curve is needed, and enables single-molecule detection. It also gives more precise and reproducible results than conventional PCR, because it overcomes the variations due to variable matrix effects in calibrators and test samples. No calibrators are required in this technology, while the analysis is based on the separation of the sample, after dilution, into a large number of partitions (droplets), which are separately analyzed. This technology has been applied to food authentication for the identification of meat-specific DNA sequences [66, 76, 77].

Conclusions

A review for DNA-based analytical techniques for milk authenticity testing is here reported. New analytical methods for adulteration detection of dairy products that offer high multiplicity and high-throughput are of great demand because of the increased incidents of substitution of milk in milk-based products with milk of lower nutrition value and quality to gain more profit. Apart from the protection of the consumers from fraud, many health risks are also of significant concern (e.g., allergies). This review reports the analytical methods that have been developed and based on the analysis of animal-specific DNA sequences. DNA is the most appropriate analyte compared to proteins mainly due to its thermal stability in processed food products. The techniques that involve hybridization assays with species-specific oligonucleotide probes present the most advantages such as the identification of the PCR-amplified DNA sequences and the lower detection limits. Hybridization assays on solid supports have also been tested giving the potential of increased multiplicity and applicability of the method that means increasing the number of different animal-species that can be simultaneously identified. Finally, the detectability and specificity of PCR-based methods was depended, apart from the applied technique, on the DNA sequence that was chosen to serve as DNA target, with mitochondrial DNA to be a more efficient target than genomic DNA, as well as the sequence of the specific primers that were used for DNA amplification.

Compliance with ethical standards

Conflict of interest The author declares that she has no conflict of interest.

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