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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE DETECTION OF COW AND DONKEY MILK

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Abstract

A real-time PCR allelic discrimination TaqMan assay based on the analysis of a single nucleotide polymorphism enabling the differentiation of cow (*Bos taurus*) and donkey (*Equus asinus*) milk was developed. Specific primers and probes were designed on the mitochondrial cytochrome c oxidase subunit I gene. The primers were designed upstream and downstream the chosen diagnosis site in a conserved region. Two probes were designed to specifically hybridise to B. taurus and E. asinus sequences. The test allowed the discrimination of bovine and donkey DNA in all blood and pure milk samples giving an unambiguous result plot of rapid and easy interpretation. The detection threshold was 2 % of cow milk in donkey milk. The applicability of the method to matrices containing degraded DNA was demonstrated by analyzing samples of raw donkey and cow milk autoclave-treated (121°C for 15 min). Finally, the assay when applied to milk samples collected from the retail trade has confirmed the species indicated in the label. Furthermore, the assay represents a potentially valuable diagnostic tool for species identification in dairy products for allergic people.

Keywords: Cow milk protein allergy, Donkey milk, Species identification, Real-time PCR allelic discrimination

Introduction

In the last few years, a considerable number of children below the age of 3 result allergic to cow's milk protein [1]. To cope with this problem, the most applied strategy is the replacement of cow milk with milk from other mammalian. Sheep's and goat's milk have proved unsuitable due to the clear cross-reactivity of their protein with bovine protein [2, 3]. On the contrary, mare and donkey milk are much more similar to human milk in composition and palatability. Unfortunately, mare's milk is difficult to find and to collect, while donkey's milk seems more available and therefore it could represent an ideal food for allergic children [4–7]. On the other hand, its high price could induce some fraudulent practices, such as the addition of less expensive milk (cow's, goat's or sheep's), so nullifying its therapeutic function. For these reasons, the development of analytical methods for an unmistakable identification of the species origin of milk and dairy products makes nowadays the object of numerous investigations [8, 9]. In this field, two different approaches are generally carried out: (1) the analysis of protein and (2) the application of biomolecular techniques. With regard to protein analysis, capillary electrophoresis [10], 2D electrophoresis, HPLC [11, 12], ELISA [13, 14] and isoelectric focusing of milk caseins [15], which is accepted as a reference tool for cows' milk detection [16], are widely used. More recently, however, this analytical approach has been largely replaced by the DNA-based analysis, which is sensitive, reliable and allows to process even very small amounts of sample [17]. Nowadays, the most applied biomolecular techniques are PCR-restriction fragment length polymorphism (PCRRFLP) [18, 19], multiplex PCR [17, 20, 21] and real-time PCR [22–25]. Both fluorescent (real-time PCR-based) and non-fluorescent (PCR and PCR-RFLP) systems have several advantages: multiplex PCR assay can be used to detect different species by means of a single reaction; PCR-RFLP analysis can be used to identify closely related species, and real-time PCR does not require any post-amplification step and has a high automation potential, allowing the processing of large numbers of samples. The aim of the present study was to develop a fluorescent qualitative test for the identification of bovine and donkey DNA in milk. The assay proposed is based on the analysis of a diagnosis site by means of a real-time PCR allelic discrimination TaqMan minor groove binding (MGB) test.

Materials and methods

Samples

Ten donkey (*Equus asinus*) and 10 bovine (*Bos taurus*) blood samples were used as positive control to set up the assay. Ten samples of raw donkey milk and ten samples of raw cow milk were obtained directly from farms. In addition, to evaluate the detection limit threshold, one cow and one donkey raw milk samples were subjected to somatic cell count (Somacount 150, Bentley Instruments) and diluted in phosphate-buffered saline (PBS) for normalisation. Successively, mixtures containing defined percentage of the milk of two species were prepared: 50, 30, 20, 10, 4, 2, 0.4 and 0.2 % of cow milk in donkey milk. The analytical sensitivity of each MGB dual-probe assay was firstly tested at least three times for each serial dilution. Finally, to evaluate the applicability of the method to matrices containing degraded DNA, 10 samples of raw donkey and cow milk were autoclave-treated (121 °C for 15 min) and ten donkey pasteurised milk samples were purchased at the local supermarket.

DNA extraction

To extract the DNA from blood, 10 ml of heparinised samples was subjected to centrifugation at 3,0009g for 20 min and 1 ml of buffy coat was withdrawn and stored at -20 °C. DNA was extracted from 50 ll of buffy coat using DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol for cultured cells. Milk samples (1 ml) were centrifuged at 1,5009g for 15 min to collect somatic cells. The pellets were rinsed three times in 1 ml of PBS, centrifuged at 12,0009g for 5 min and finally resuspended in 200 ll of PBS. DNA was extracted following the protocol of DNeasy Blood and tissue kit (Qiagen) following the manufacturer's protocol for tissue samples. DNA was quantified using the NanoDrop-2000 (Thermo Fisher Scientific).

Primer and probe design

The cytochrome c oxidase subunit I (CoxI) sequences obtained from the GenBank database, corresponding to *B. taurus* (GenBank Accession No. NC006853, EU177865, EU177866) and *E. asinus* (GenBank Accession No. NC001788, AP012271, X97337), were aligned with the ClustalW software [26] for the detection of the polymorphic site to be used as diagnosis position. All the sequences available for each species were examined to confirm the absence of intraspecific variation in the chosen diagnosis site (Table 1). Based upon these sequence alignments, we designed a TaqMan MGB allelic discrimination assay around the polymorphic site using Primer Express software (Applied Biosystems). Forward (5'-TGATTCCCACTATTCTCAGG-3') and reverse (5'-GTGTTGTGGGAAGAAGGTTATA-3') primers were designed in a conserved region both in *B. taurus* and in *E. asinus*, upstream and downstream the chosen diagnosis site (Table 1). One probe was designed to specifically hybridise, with 100 % of homology, to the *B. taurus* sequence (5'-6FAMTGAGCCAAAATCCACTT-3'-MGB) and the other was designed to hybridise, with 100 % of homology, to the *E. asinus* sequence (5'-VIC-TGAGCAAAAATCCACTT-3'-MGB). The two probes have 100 % homology with *B. Taurus* and *E. asinus* sequences, while a single mismatch in proximity of 5'end, corresponding to diagnosis site, was observed between them. Primers and probes were synthesised by Applied Biosystems. Analysis of diagnosis site Real-time PCR was performed in a 25 µl reaction mixture containing 900 nM of both forward and reverse primers, 200 nM of each probe, 12.5 µl TaqMan Genotyping Master Mix (Applied Biosystems) and 10 ng of DNA template. Thermal cycling was performed on a 7300 real-time PCR System (Applied Biosystems) under the following conditions: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analysed in triplicate and real-time PCR was executed in triplicate. During amplification, the simultaneous presence of the two probes allows the annealing of both regardless of the DNA present (donkey/bovine), because the single mismatch does not impair the probes' annealing to heterologous DNA. During the extension, the 5'–3' exonuclease activity of the DNA polymerase degrades the probe that has 100 % of homology with the template with a consequent fluorescence emission. On the contrary, the probe having a mismatch with the sequence is removed without degradation and no fluorescence emission. The post-PCR analysis allows the instantaneous visualisation of the results by means of a specific plot. Results were obtained by the automatic calling feature of the allelic discrimination option in SDS v1.2.1 software (Applied Biosystems). Species were assigned to samples by visually inspecting a plot of the fluorescence (Rn) from the donkey probe versus the Rn from the bovine probe generated from the post-PCR fluorescence read.

Statistical analysis

The degree of agreement between replicates within the same run (repeatability) or between replicates tested by different persons (reproducibility) was measured. To evaluate the accuracy of the developed test, inter and intra-assay variations were calculated by means of coefficient of variation (CV) of both alleles.

Results and discussion

Nowadays, consumers are more and more interested in the topic of wholesomeness and authenticity of food, so the identification of the animal species is gaining increasing importance. Therefore, a great number of analytical techniques have been developed and proposed for this purpose. At present, the biomolecular methods seem to be the most promising. They involve the use of species-specific primers when the target sequences present an adequate number of mismatches. Alternatively, when the number of mismatches is not high, the analysis of a single nucleotide polymorphism (SNP) is preferable. A widely applied technique is PCR-RFLP, in which DNA amplified by PCR is digested with restriction endonucleases. This technique has been widely used for species identification in dairy products [18, 19]. However, when mixtures of two species are to be detected, the interpretation of results could be difficult due to possible overlapping restriction patterns [21]. Furthermore, the secondary conformation can prevent the digestion of PCR products by restriction endonucleases. The consequent presence of indigested products causes a misinterpretation of the results [27]. These difficulties have directed the research towards alternative methods for the analysis of SNP. Multiplex primer extension reaction has been successfully applied in discriminating many animal and bacterial species in different food items [28–31]. However, some problems could arise when mixed matrices are considered. On the contrary, the real-time PCR allelic discrimination test has been successfully proposed for the identification of cows' and buffalos' milk [32] also in mixed matrices. In the past, this technique has been largely used in genetic and microbiological studies [33, 34]. In our study, we developed a real-time PCR allelic discrimination TaqMan MGB assay based on a diagnosis site analysis for the identification of donkey and bovine milk. Analysis of the alignment of the reference sequences obtained from GenBank showed that the base in 6915 position (referred to GenBank Accession No. NC006853) of CoxI gene could differentiate between *B. taurus* and *E. asinus* (Table 1). This diagnosis site did not show any intraspecific variation when compared with GenBank sequences for each species. The specificity of real-time PCR allelic discrimination TaqMan assay described in this study was demonstrated in all blood and milk samples (Fig. 1; Table 2). In Fig. 1, each data point represents the normalized endpoint fluorescence reading for both VIC- and FAM labelled probes plotted on the x- and y-axes, respectively. The plot shows that there is unambiguous discrimination between species with two distinct populations. One population clusters around the x-axis and consists of *E. asinus* samples. The second population clustering around the y-axis represents B. taurus samples. The specificity of the test of the distinctly performed tests implying a perfect 'analytical' repeatability and reproducibility of the assay. Triplicate execution of the PCR on the samples in fact provides an estimate of the practical repeatability. For as concern the intra-assay variations, the coefficient variations were 0.038 (CV max was 0.093) and 0.028 (CV max was 0.071) for allele X and allele Y, respectively. For as concern the inter-assay variations, the coefficient variations were 0.041 for both alleles. For as concern the detection limit threshold, the assay consistently revealed the addition of cows' milk to donkey milk as low as 2 % (Fig. 2; Table 3). However, the possibility of detecting small amounts of contaminating milk can represent a

problem in certain circumstances because it is difficult to distinguish inadvertent cross-contamination from fraudulent practices. It was possible to confirm the species in all autoclave treated milk samples, so demonstrating that the test could be applied to matrices containing degraded DNA (Table 2). The small size of the amplified fragment (102 bp) allows application of the test to heat-treated products. In fact, thermal processing such as UHT procedure or sterilization breaks the DNA in fragment of about 300 bp long [28]. Finally, the applicability of the method was demonstrated: it was possible to confirm the species indicated in label in all donkey milk samples collected from the retail trade (Table 2). In conclusion, the assay described in this paper provides advantage over (1) the PCR-RFLP, as it can be applied to mixed matrices and (2) the traditional PCR assays, because it eliminates the need for post-PCR processing step, such as gel electrophoresis and ethidium bromide staining. Moreover, since the real-time PCR allelic discrimination TaqMan test does not require any post-amplification step and it utilises 96-well format plates that can be read in about 5 min, it enables a rapid diagnosis of large numbers of samples. Finally, fluorescent real-time PCR includes both amplification and sequence-specific molecular verification, excluding the risk of false-positives due to a misamplification. In conclusion, our results suggest that this real-time PCR allelic discrimination assay proposed is a reliable way to identify and differentiate between *B. taurus* and *E. asinus* and represents a potentially valuable diagnostic tool for species identification in dairy products for allergic people.

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Table 1

Primer and probe design.

Species	Genbank Acc. N.	Aligned sequences 5′---- 3′
Bos taurus	NC006853	TTGTTCATTGATTCCCACTATTCTCAGGTTATACTCTCAACGATACATGAGCCAAAAATCC
os taurus	EU177865	.....
Bos taurus	EU177866	.....
Equus asinus	NC001788	.....C.....T.....A.....A.....TC.A..C.....A.. ....
Equus asinus	AP012271	.....C.....T.....A.....A.....TC.A..C.....A.....
Equus asinus	X97337	.....C.....T.....A.....A.....TC.A..C.....A.....
Bos taurus	NC006853	ACTTCGCAATTATATTTGTAGGCGTCAATATAACCTTCTTCCCACAACACTTTCTAGGAC
Bos taurus	EU177865	.....
Bos taurus	EU177866	.....
Equus asinus	NC001788	....TA.....C.....G.....T.....C..T..C.
Equus asinus	AP012271	....TA.....C.....G.....T.....C..T..C.
Equus asinus	X97337	....TA.....C.....G.....T.....C..T..C.

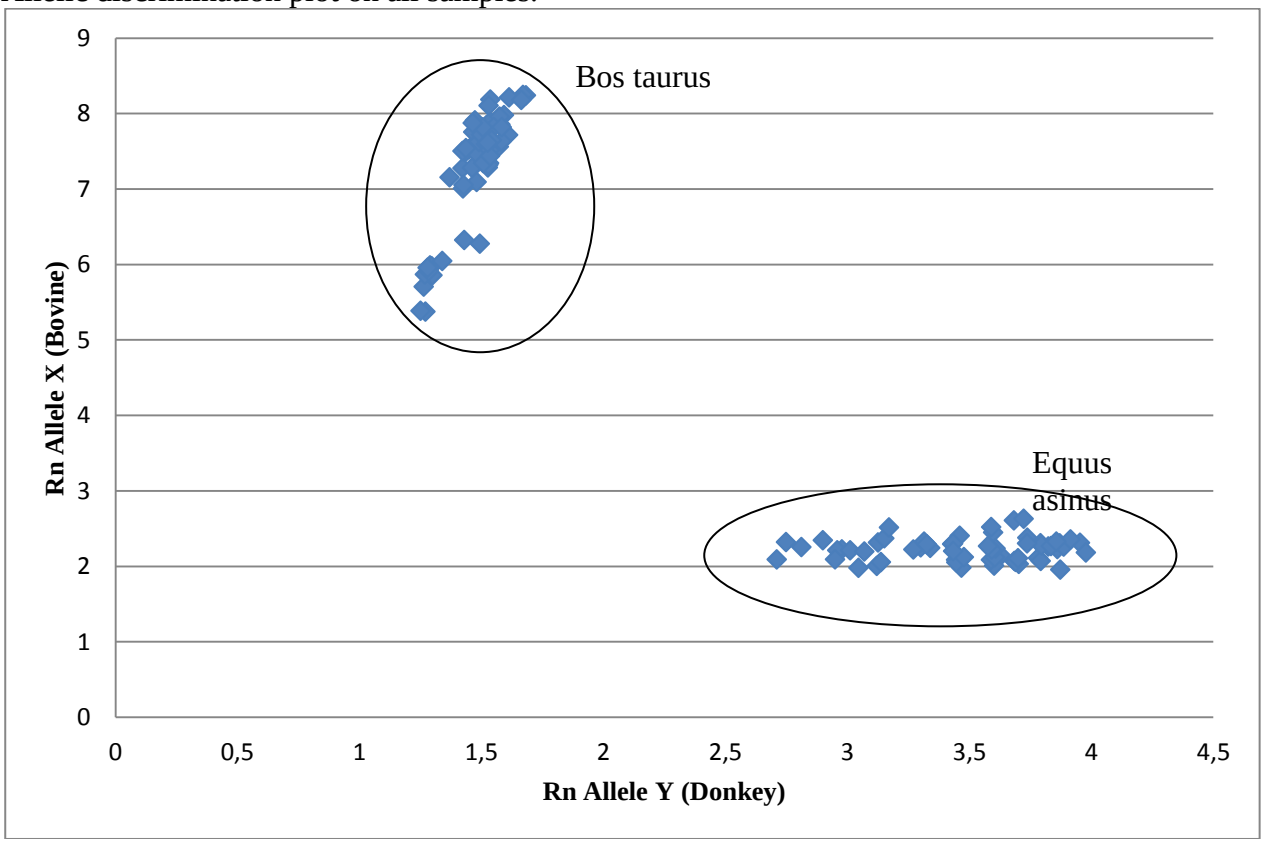
Primers binding site for primers and probes (underlined). The bases highlighted correspond to the diagnosis site.

Table 2
Results on all samples.

Samples	Species	Rn allele Y (media±SD)	Rn allele X (media±SD)	Delta (Y-X)	Ratio (Y/X)	TaqMan result
Blood	*Equus asinus* (n=10)	3.43±0.33	2.12±0.11	1.31±0.40	1.63±0.31	A
	Bos taurus (n=10)	1,44±0.13	6.93±0.96	-5.50±0.85	0.21±0.16	C
Raw milk	*Equus asinus* (n=10)	3.36±0.32	2.14±0.13	1.20±0.32	1.57±0.16	A
	Bos taurus (n=10)	1.53±0.08	7.64±0.36	-6.10±0.31	0.20±0.01	C
Autoclaved milk	*Equus asinus* (n=10)	3.58±0.35	2.29±0.05	1.29±0.38	1.57±0.17	A
	Bos taurus (n=10)	1.43±0.11	6.96±0.93	-5.54±0.82	0.20±0.01	C
Commercial milk[a]	*Equus asinus* (n=10)	3.54±0.33	2.36±0.14	1.17±0.34	1.50±0.15	A

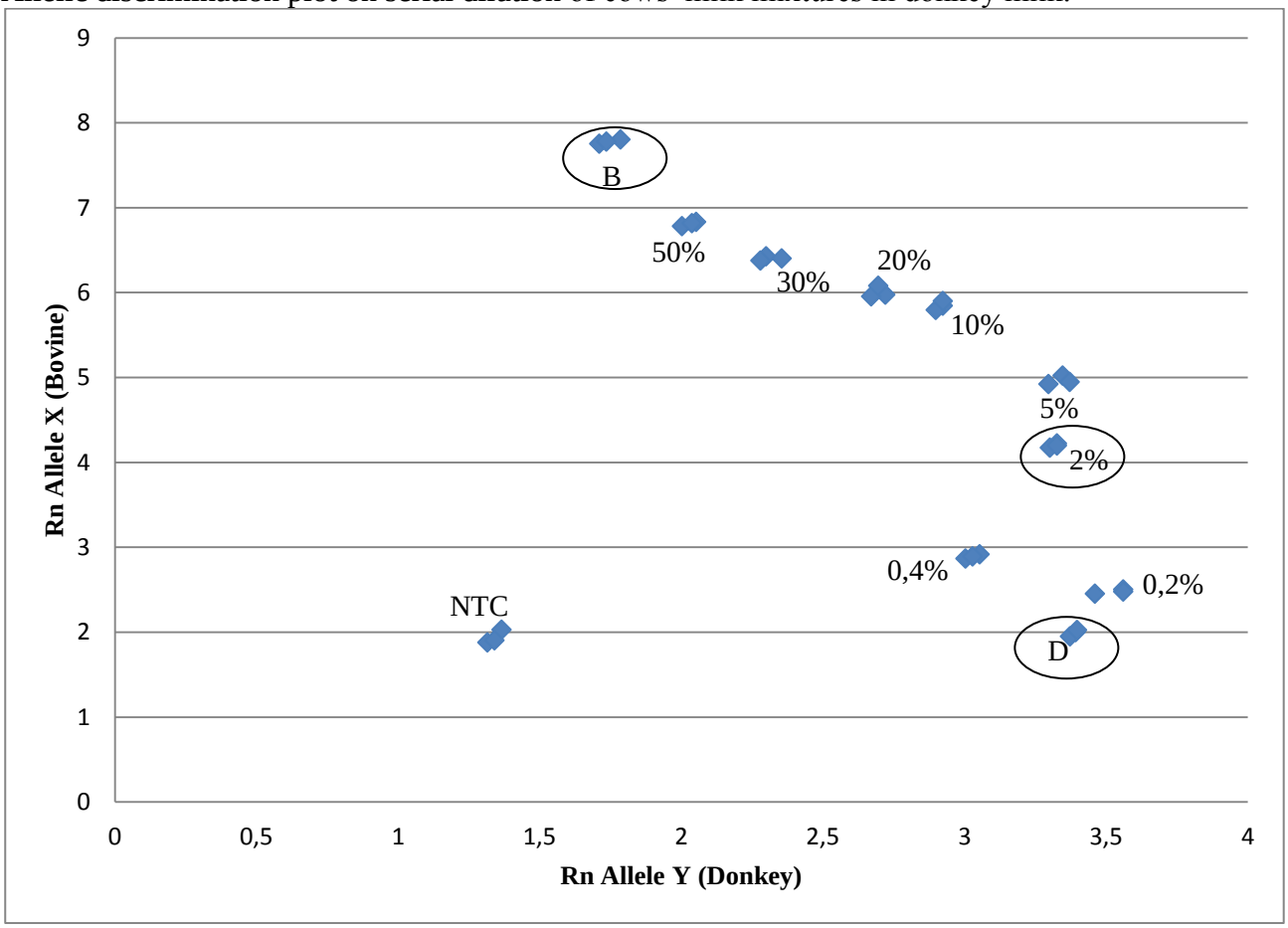
[a] Commercial samples originated from different italian regions: Lombardia (n=1), Lazio (n=1), Abruzzo (n=1), Basilicata (n=2), Calabria (n=2) and Puglia (n=3).

Figure 1
Allelic discrimination plot on all samples.



Real-time Allelic discrimination test on all samples. The plot shows the fluorescence of the donkey (x-axis) and bovine (y-axis) probes.

Figure 2
Allelic discrimination plot on serial dilution of cows' milk mixtures in donkey milk.



Real-time PCR Allelic Discrimination test on dilutions of raw cows' milk mixtures in raw donkey milk. The plot shows the fluorescence of the donkey (x-axis) and cow (y-axis) probes.