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CASE REPORT

Bite through the tent

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Abstract The authors report on a young boy who was bitten into his face by an unknown animal while being asleep in a tent. Given the bite marks and the location of the scene, members of the mustelidae and canidae families were the first "suspects." Deoxyribunucleic acid (DNA) recovered from the tent's wall was analyzed with regard to parts of the mitochondrial 12S ribosomal ribunucleic acid (12S rRNA) and cytochrome b (cytb) genes as well as nuclear short tandem repeats (STRs). Since Sanger sequencing revealed a mixed sequence with a strong human component overlying the nonhuman contributor, an animal screening using a duplex real-time polymerase chain reaction (PCR) with an intercalating dye and melt curve analysis was employed. The results were later confirmed by cloning. The applied commercial canine STR kit verified the animal family (canidae) but did not help in discriminating the species due to cross-species amplification. In the presented case, the real-time PCR assay offered the cheapest and fastest method for animal family determination, which then allowed for an appropriate and sample-saving strategy to characterize the causative animal species.

Keywords Animal species · DNA mixtures · Mitochondrial DNA · Real-time PCR · Melt curve analysis · Sanger sequencing

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Introduction

The determination of animal families or species is of interest to forensic genetics, especially when related to the prosecution of wildlife crimes [1]. Analysis of mitochondrial deoxyribunucleic acid (mtDNA) is the method of choice due to a higher success rate processing low copy number or degraded DNA samples as well as to its arrangement of conserved and specific regions. For species determination, the mitochondrial cytochrome b (cytb) [2, 3] and 12S ribosomal ribunucleic acid (12S rRNA) [4–6] genes proved useful together with cytochrome I oxidase and 16S ribosomal RNA genes [1, 7]. The most common approach in sporadic forensic species determination is Sanger sequencing. However, the interpretation of DNA mixtures may be difficult. Here, we present a case that was solved employing an animal family-screening assay based on real-time polymerase chain reaction (PCR) and melt curve analysis prior to species-specific PCR.

Case report

On a summer morning, a 7-year-old boy was found by his parents with bleeding wounds on his cheeks. The boy had slept overnight in the family's tent that was set up in their garden next to a hedge. Since it was a very hot summer, only

the inner tent had been set up, without the outer tent. Obviously, something had grabbed the boy's face while he was asleep and snuggled tightly to the tent's wall. Upon examination, characteristic bite marks were found on the boy's face and in the tent's wall (Fig. 1). No further indications on the causative animal were present at the scene. The morphology of the bite marks and the surrounding area (garden near a wood) suggested a representative from the canidae or mustelidae families as first suspects, in special, a badger was suspected. The boy's wounds were medically treated, and a preventive rabies vaccination was given.

The part of the tent's wall showing the bite marks was cut and sent to the Institute of Legal Medicine for further identification of the animal species.

Materials and methods

All commercially available kits were used according to the manufacturers' protocols unless stated otherwise.

Samples, DNA extraction, and quantification

The tent's wall showed several characteristically arranged holes up to 3 mm in diameter, partly accompanied by saliva traces. Two small pieces of fabric were processed completely, and also three swabs (Sarstedt, Nümbrecht, Germany) were taken from the saliva traces. DNA was extracted using the MN tissue DNA extraction kit (Machery Nagel, Düren, Germany). Total DNA amount was quantified using the Qubit v2.0 (Life Technologies, NY, USA).

PCR conditions

Universal PCR

A part of the 12S rRNA gene was amplified in 10-µl volumes containing 10×PCR reaction buffer (Applied Biosystems, Foster City, CA, USA), 200 µM of each dNTP, universal primers (Biomers, Ulm, Germany; cf. Table 1), 1 U AmpliTaq Gold (Applied Biosystems) and 1 µl of extracted DNA. Cycling protocol was 95 C for 10 min, 32 cycles with 95°C for 15 s, 54°C for 30 s, 72°C for 90 s, final extension at 72°C for 10 min. PCR reactions were purified either by adding 2 µl ExoSap-IT (USB, Cleveland, OH, USA) followed by incubation at 37°C for 30 min and 80°C for 15 min, or by using the QIAquick® Kit (Qiagen, Hilden, Germany).

Species-specific PCR

PCR with primers specific to fox and dog 12S rRNA was setup as mentioned above but in a final volume of 5 µl, and with modified denaturation (45 s), annealing (64°C), and elongation (30 s). The whole reaction was used for agarose gel analysis. DNA from dog (500 pg), fox (500 pg), and human (5 ng) were amplified as positive controls and to monitor cross-specificity.

Sanger sequencing and sequence analysis

The BigDye™ v1.1 Terminator kit (Applied Biosystems) was used for direct sequencing in final volumes of 5 µl consisting of 5×BD v1.1 Terminator Ready Reaction Mix, 5×BD Dye Terminator Sequencing Buffer, 0.2 µM PCR primer or M13 primer, respectively, and 1 – 3 µl of PCR product or 1 µl of plasmid DNA. After purification of the sequencing reaction products (DyeEx96 column plates or DyeEx2 columns; both: Qiagen), capillary electrophoresis (POP6, 36 cm capillary) was either run on a 3130*xl* or on a 3100*Avant* Genetic Analyzer (Applied Biosystems) with Foundation Data Collection Software v3.0 and v2.0, respectively. The obtained sequences were blasted against the NCBI database. Sequencher 4.9 software (GeneCodes, Ann Arbor, MI, USA) was used to compare the results to the 12S rRNA and cytb regions of the human (NC_012920), dog (NC_002008.4), and fox (NC_008434) NCBI mitochondrion reference sequences.

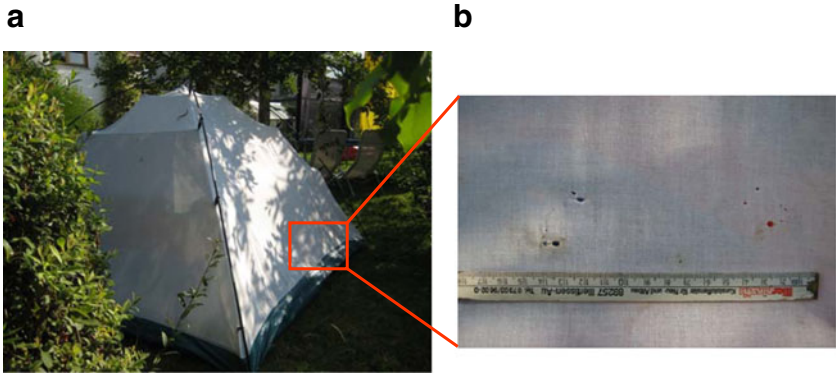

Fig. 1 Bite marks on the tent. **a** Situation of the inner tent in the garden; **b** tent wall showing the bite marks

Table 1 Primers used

Target	Species	Used for	Direction	Sequence	Concentration (nM)	$MgCl_2$ (mM)
12S rRNA	Universal	PCR, sequencing, cloning	Forward*	CCCCACGGGAAACAGCAGT	200	1.5
			Reverse**	TAGAACAGGCTCCTCTAG	200	
12S rRNA	Human	Screening assay	Forward	AGTGTTTTAGATCACCCCCTCCCC	300	2.5
			Reverse	ATGTTAAAGCCACTTTCGTAGT	200	
cytb	Human	Screening assay	Forward	GGCCACAGTAATTACAAACTTACTATC	100	
			Reverse	TGTGAGGGTGGGACTGTCTACTGAGTA	100	
12S rRNA	Mustelidae	Screening assay	Forward	ACGGCGTAAAACGTGTTAAG	50	2
			Reverse	TATTAACAGTAGCTTTTTACGGCCT	50	
cytb	Mustelidae	Screening assay	Forward	GCAACCGTAATTACCAACTTACTGTC/ GCAACCGTAATTACTAATTTACTATC	50 each	
			Reverse	ATGAATGGTAAGATGAAGTGGAA/ ATGAATGGCAGGATAAAGTGGAA	50 each	
12S rRNA	Canidae	Screening assay	Forward	ACTAAAGTTAAAACTTAACTAAGCCGTA	300	1.5
			Reverse	ATCGTGTAGTCAGATTATTATAAAGTCAC/ CGTGTAATCAGAAAATTTTAAAGTCAC	300 each	
cytb	Canidae	Screening assay	Forward	ACTAATCTTCTCTCTGCCATCCC/ ACAAATCTTCTATCTGCTATCCC	50 each	
			Reverse	AATCGTGTTAGGGTTGCTTTGTC	50	
12S rRNA	Dog	Dog-specific PCR	Forward	CGTAAAGCGTGTTCAAGATACTTTTAC	250	1.5
			Reverse	ATCGTGTAGTCAGATTATTATAAAGTCAC (as in screening assay)	250	
12S rRNA	Fox	Fox-specific PCR	Forward	CGTAAAGCGTGTTTAAGATAACATATT	250	1.5
			Reverse	CGTGTAATCAGAAAATTTTAAAGTCAC (as in screening assay)	250	

*Taken from: Karlsson and Holmlund [8]

**Taken from: Balitzki–Korte et al. [5]

Screening assay: real-time PCR and melt curve analysis

Real-time PCR reactions were performed in 5-µl volumes containing 2.5× qPCR GreenMaster Mix including the intercalating dye EvaGreen (Jena Bioscience, Jena, Germany), 0.1 µl of 1.25× ROX (Jena Bioscience), 1.5–2.5 mM $MgCl_2$ (cf. Table 1), 12S rRNA and cytb primers (cf. Table 1) specific to either canidae, mustelidae, or human, respectively, and 1 µl of extracted DNA. Reactions were cycled on an AB7500 with 7500 System SDS software v1.2.3 (Applied Biosystems): 95°C for 10 min, 30 cycles with 95°C for 45 s, and 66°C for 60 s, followed by a continuous melt curve analysis from 60 to 95°C using default instrument settings.

Canine STRs

The StockMarks Canine short tandem repeat (STR) kit (Applied Biosystems) was used and capillary electrophoresis was run on a 3130*xl* Genetic Analyzer (Applied Biosystems). Data was analyzed with GeneMapper IDX (Applied Biosystems) following instructions provided with the kit.

Cloning of mixed DNA samples

After purification (QIAquick® Kit, Qiagen), 12S rRNA fragments with mixed sequences were ligated into a pCR4-TOPO TA cloning vector using the FastPlasmid Cloning kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Plasmid DNA from 20 clones was prepared with the Perfectprep Plasmid 96VAC Kit (5Prime, Hamburg, Germany). Both strands were sequenced using M13 primers.

Results

Universal PCR and direct sequencing

Universal 12S rRNA primers were employed in all five DNA samples. PCR from one of the swabs failed. The remaining four samples showed mixtures of human DNA and another source (example given in Fig. 2). It was not possible to "extract" the underlying nonhuman sequence in order to obtain well-grounded information about the second contributor.

Screening assay

The screening assay specific to mustelidae excluded an animal from this family as DNA source (Fig. 3a). Regarding the fabric samples, the assays specific to *Homo sapiens* (Fig. 3b) and to canidae (Fig. 3c) both showed clear signals. In the melt curve analysis, peaks were observed corresponding to the T_m of the respective 12S rRNA (first peak) and cytb (second peak) PCR fragments. However, the swabbed samples showed only signals for human but not for the canidae.

PCR with dog-/fox-specific primers

For further discrimination within the family of canidae, primers specific to the 12S rRNA regions of dog as well as of fox were designed. As visualized by agarose gel electrophoresis (Fig. 3d), only the fox-specific primers yielded a PCR product. Fox, dog, and human positive controls showed the expected results excluding cross-species amplification.

STR results

The StockMarks Canine STR kit revealed an incomplete STR profile (Fig. 4a). Cross-specificity to the vulpini tribe within the canidae family was depicted by the positive controls (fox DNA: Fig. 4b; wolf DNA: Fig. 4c; dog DNA: Fig. 4d). However, the fox and wolf samples showed several peaks outside of the defined allele ranges.

Cloning of PCR fragments

The sample with the highest ratio of the second sequence contributor was chosen for confirmative cloning. Analysis was successful in 15 out of 20 clones picked. Of these, 12 clones showed a human 12S rRNA sequence, and three showed a fox 12S rRNA sequence. No sequences indicating another nonhuman contributor were found.

Discussion

In the presented case, application of the standard procedure in sporadic forensic animal species determination (PCR with

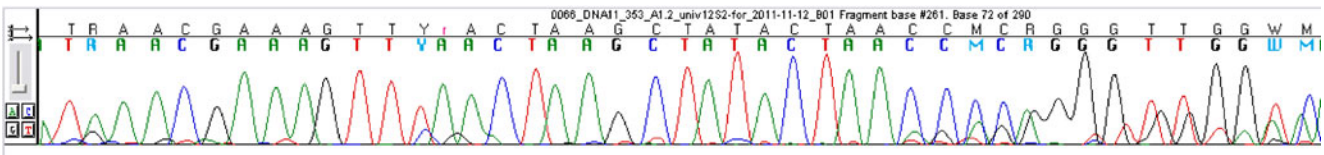

Fig. 2 Mixed sequence as obtained from the 12S rRNA universal PCR. The sequence shift observed in the right half of the pane was later explained by a 2 bp deletion in the fox 12S rRNA sequence

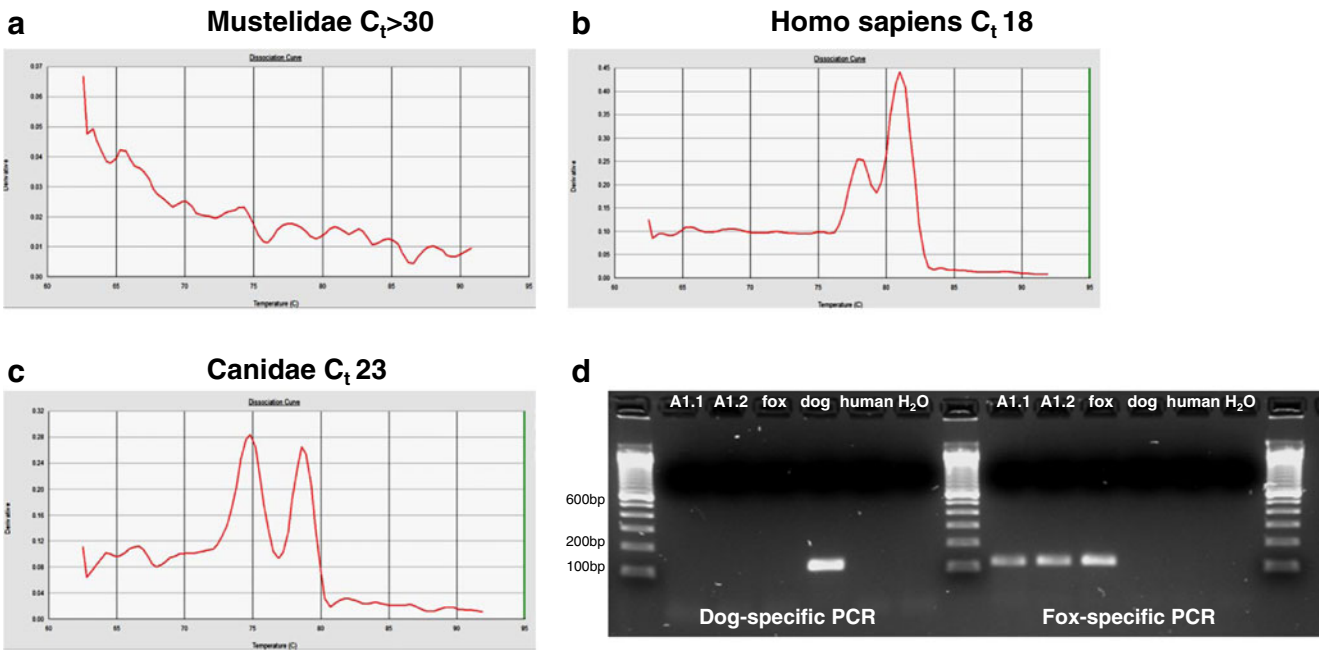

Fig. 3 Family-specific duplex real-time PCR assay (12S rRNA, cytochrome b) with melt curve analysis, and species-specific 12S rRNA PCR. **a**–**c** Derivative melt curves as resulting from the duplex reaction detecting 12S rRNA (first peak) and cytb (second peak); **d** dog- and fox-specific 12S rRNA PCR employed in two of the samples (A1.1 and A1.2)

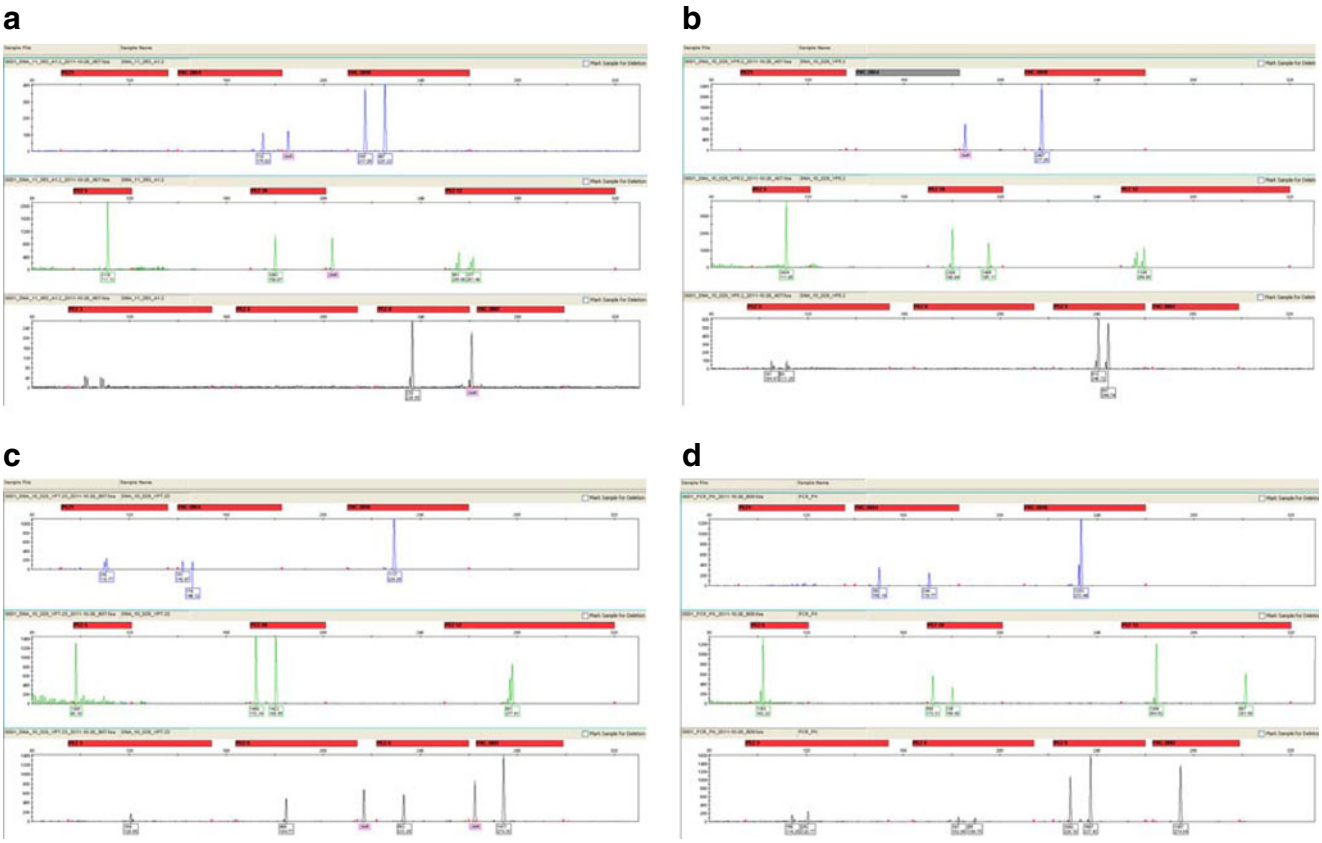

Fig. 4 STR analysis employing a commercial canine STR kit. Cross-(sub)species amplification in three members of the canidae family. **a** Case sample; **b** red fox (vulpini tribe); **c** wolf (canini tribe); **d** dog (canini tribe; control DNA included in the kit)

universal primers and sequencing) revealed a mixed nonhuman/human sequence with a predominant human component. In such a case, species determination may fail unless more sophisticated and time-consuming methods like cloning are employed. Our screening assay disclosed a canidae source DNA that was then further determined as DNA of a fox by specific PCR. The supposed "shortcut" by applying a canine STR kit failed due to cross-specificity within the canidae family (wolf, dog, and red fox tested) leading to a STR profile in the fox sample.

Considering the circumstances of the case, the screening was restricted to mustelidae, canidae, and *H. sapiens*. After rapid exclusion of a mustelidae source DNA (suspected badger), the decision was made to further discriminate the canidae source DNA due to the legal implications of the case: if a dog's DNA would have been detected, then prosecution of the dog owner might have been the consequence after assuring the dog's identity by a standardized individualizing analysis [9, 10]. A family screening prior to species determination also seems reasonable whenever there is a limited amount of sample DNA. Using an intercalating dye instead of labeled probes decreases costs and proved to be adequately specific. As the primers for the screening assay are designed to cover several species within a given family, the assay's sensitivity varies depending on the species actually detected. A detailed study on the screening assay's components' sensitivities is in progress.

Resolving DNA mixtures can be problematic. Due to the meshy structure of the tent's fabric, there was no advantage in trying to swab the saliva traces from the surface vs. processing a complete cut-out of fabric in this case. In fact, the swabbed samples showed only the human but not the canidae component, possibly due to the generally lower amount of DNA recovered by swabbing. However, swabbing has proven effective on plain and non-soaking surfaces [11].

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