

Identification of canine saliva using mRNA-based assay

Hiroaki Nakanishi¹ · Takeshi Ohmori² · Masaaki Hara³ · Katsumi Yoneyama³ · Aya Takada³ · Kazuyuki Saito¹

Received: 18 October 2015 / Accepted: 18 May 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract A dog saliva analysis in addition to a bite-mark analysis may be important for evidence when a crime involves a dog bite. In this study, the utility of detecting canine saliva-specific mRNAs to identify canine saliva was evaluated. Canine saliva swabs ($n=20$), urine swabs ($n=20$), body surface swabs ($n=20$), whole blood samples ($n=10$), human saliva ($n=20$), human skin surface swabs ($n=20$), and human whole blood ($n=20$) were tested. The saliva-specific genes encoding statherin (STATH), carbonic anhydrase VI (CA-VI), and dog allergens (Canf1 and Canf2) were analyzed as candidate genes. Moreover, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as confirmation of canine mRNA extraction. STATH, CA-VI, Canf1, Canf2, and GAPDH mRNAs were detected in 19/20, 1/20, 11/20, 4/20, and 20/20 saliva samples, respectively. The STATH, CA-VI, Canf1, Canf2, and GAPDH mRNAs did not exhibit cross-reactivity with samples of human origin. This mRNA-based assay was also able to detect canine saliva in mock forensic samples. The results of this study indicated that the detection of STATH mRNA is useful for the identification of canine saliva, and GAPDH is a suitable marker for canine mRNA extraction.

Keywords Canine saliva · mRNA · Statherin · Carbonic anhydrase VI · Canf1 · Canf2

Introduction

Dogs have been bred by many cultures globally from ancient times as guards, hounds, and house pets. Some individuals that breed dogs as pets have a large-sized dog or a species of quick-tempered dog. Owners of such pets can occasionally get into trouble with neighbors. In particular, injury to anyone by a dog bite may result in a criminal case. From 1979 to 2005, dogs bit more than four million people in USA each year, resulting in the hospitalization of 6000 to 13,000 people, and a mean of 19 deaths were reported annually from dog attacks [1]. More than half of US states have a strict liability statute that makes the dog owner fully liable if their dog bites someone [2]. The identification of bite marks and DNA identification of the dog are evidentially important in any crime involving a dog bite. Various methods of canine DNA identification have been developed [3–5], and their application in real-world forensic practice reported [3, 4]. Conversely, analysis of bite marks generally has been investigated as the identification method for dog bites [6–8]. However, Bernitz et al. [8] warned against over interpreting poor-quality bite marks because dog bite marks tend to be distorted. Identifying a canine bite mark might be difficult for a non-specialist, and the damage caused by a bite, such as repeated bites, might be too complex for even a specialist to identify. Canine saliva adheres to human skin when a dog bites. In addition to a bite-mark analysis, if a method to identify canine saliva was developed, it may be more reliable to identify a dog bite. However, no such method has been reported. Human saliva is estimated by detecting of α -amylase [9–11], but this test cannot be used to estimate canine saliva because of the low level of α -amylase activity

✉ Hiroaki Nakanishi
hnakani@juntendo.ac.jp

¹ Department of Forensic Medicine, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-Ku, Tokyo 113-8421, Japan

² National Research Institute of Police Science, 6-3-1 Kashiwanoha, Kashiwa, Chiba 277-0882, Japan

³ Department of Forensic Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Saitama 350-0495, Japan

in the canine parotid [12]. Moreover, no anti-canine saliva antibody is commercially available.

To identify human saliva, mRNA-based assays targeting genes expressed specifically in saliva have been developed [13–15]. We investigated whether this technique could be applied to the identification of canine saliva. Kamata et al. [16] reported that mRNAs of Canf1 and Canf2, canine allergens, were secreted in canine saliva. Moreover, Kasuya et al. [17, 18] reported that carbonic anhydrase VI (CA-VI) was secreted in canine saliva, and they determined its cDNA sequence [19]. The mRNA-based identification of human saliva by detecting the saliva-specific marker statherin (STATH) is useful [13–15]. Therefore, it is possible that canine STATH mRNA could function as a marker for the identification of canine saliva. We hypothesized that mRNA expression of these genes would be specific for canine saliva.

Our aim was to develop a method for the identification of canine saliva by detecting the expression of specific mRNAs. In this study, we evaluated the utility of Canf1, Canf2, CA-VI, and STATH mRNAs in the identification of canine saliva.

Materials and methods

Samples

Canine saliva stain and urine stain samples were collected from 20 dogs by swabbing. Canine body surface samples were collected from 20 dogs by wiping the skin with a wet swab; canine whole blood samples were collected from 10 dogs. Human saliva stain samples were collected from 20 donors and by swabbing. Human skin surface samples were collected from 20 donors by wiping the skin with a wet swab. Human whole blood samples were collected from 20 donors. All human and canine donors were healthy. Moreover, as forensic mock samples ($n=5$), human skin that had been bitten by a dog was sampled by wiping with a wet swab. All swab samples were dried and stored at -80°C until use, and all whole blood samples were stored at -80°C until use. Canine saliva stain samples ($n=4$) stored at room temperature for 1, 7, 14, and 28 days were used as aged samples to evaluate mRNA stability.

RNA extraction and cDNA synthesis

For total RNA extraction, a 1×1 cm piece of swab or 30 μL of whole blood was used. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands), and DNase I digestion was performed on the RNA extracts using an RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using a High-Capacity RNA-to-cDNA™ Kit (Life Technologies,

Carlsbad, CA, USA) according to the manufacturer's protocol, by addition of 9 μL of RNA to a total reaction volume of 20 μL .

Real-time PCR

The STATH, CA-VI, Canf1, and Canf2 were analyzed as candidate genes, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as confirmation of canine mRNA extraction. The 18S rRNA (18S) gene was used as confirmation of human mRNA extraction from samples of human origin. The genes were detected by real-time PCR using a minor groove-binding probe. CA-VI (Cf02622218_m1), Canf1 (Cf02622894_m1), Canf2 (Cf02622899_m1), and 18S (Hs99999901_s1) primers and probes were used with a commercially designed kit (TaqMan® Gene Expression Assay; Life Technologies). STATH and GAPDH primers and probes were designed using Primer Express® software (Life Technologies). The primer sequences are shown in Table 1. The target sites of self-designed primer were compared to all the available sequences using BLAST (www.ncbi.nlm.nih.gov/BLAST) and were confirmed as complementary to target species, but not other species.

Real-time PCR using the commercially designed kit was performed in 20- μL reaction mixtures containing 2 $\times$ TaqMan® Fast Advanced Master Mix (Life Technologies), 20 $\times$ TaqMan® Gene Expression Assay, and 2 μL of cDNA. Real-time PCR using the self-designed primers was performed in 20- μL reaction mixtures containing 2 $\times$ TaqMan® Fast Advanced Master Mix, 450 nM forward and reverse primers, 125 nM probe, and 2 μL of cDNA. PCR amplification was performed using the StepOne-Plus Real-Time PCR System (Life Technologies) programmed for 2 min at 50°C and 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . The Ct detection threshold was 0.2, at which a positive reaction for STATH, CA-VI, Canf1, Canf2, and GAPDH mRNAs was defined as a Ct value of ≤ 40 cycles, and a positive reaction for 18S mRNA detection was defined as a Ct value of ≤ 33 cycles.

Results

Testing of candidate genes

The results of the detection of four candidate mRNAs and GAPDH mRNA in various canine samples are shown in Table 2. STATH mRNA exhibited the highest detection rate (95 %) in the saliva stain samples, followed by Canf1 mRNA (55 %). In contrast, the detection rate of CA-VI mRNA was only 5 %. STATH and Canf1 mRNAs were detected in one body surface sample, but in no urine or blood samples. CA-VI mRNA was detected in 9 of 10 whole blood samples. Canf2

Table 1 Primer sequences of STATH and GAPDH

Target gene		Sequence (5'-3')	Amplicon size
STATH	Forward	CTGCTAACAGGAATCAGCCAAAT	84 bp
	Reverse	AATCAGCTCCAATCATGGCAAT	
	Probe	ATCTTTGTCTTTGCCTTTGT	
GAPDH	Forward	CCCCAATGTATCAGTTGTGGATCTG	83 bp
	Reverse	CCTGCTTCACTACCTTCTTGATGTC	
	Probe	AAAGCTGCCAAATATG	

mRNA was detected only in saliva stain samples, at a frequency of 20 %. GAPDH mRNA was detected in 11 of 20 canine urine stain samples and all canine saliva stain, skin surface, and whole blood samples. On the other hand, STATH, CA-VI, Canf1, Canf2, and GAPDH mRNA were not detected in samples of human origin although 18S mRNA was detected (18S data not shown).

Forensic application

The results of the detection of four candidate mRNAs and GAPDH mRNA in five mock forensic samples are shown in Table 2. STATH and GAPDH mRNAs were detected in all of the five mock forensic samples. In contrast, neither CA-VI nor Canf2 mRNA was detected. Canf1 mRNA was detected in one of the five forensic mock samples.

Stability of STATH and GAPDH mRNAs

The Ct values of the STATH and GAPDH mRNAs in four canine saliva stain samples stored at room temperature for 0, 1, 7, 14, and 28 day(s) are shown in Fig. 1. STATH and GAPDH mRNAs were detected in all samples. The Ct values of the canine saliva stain samples stored at room temperature for 1, 7, 14, and 28 day(s) were not significantly different from

those of corresponding fresh canine saliva samples (*t* test; $p > 0.05$).

Discussion

The α -amylase activity level in the canine parotid is 0.9 ± 0.3 U/mL [12], which is much lower than in human saliva (mean, 350 U/mL [20]), suggesting that standard methods for detecting salivary α -amylase are not suitable to detect canine saliva in salivary stains or bite marks. Therefore, the

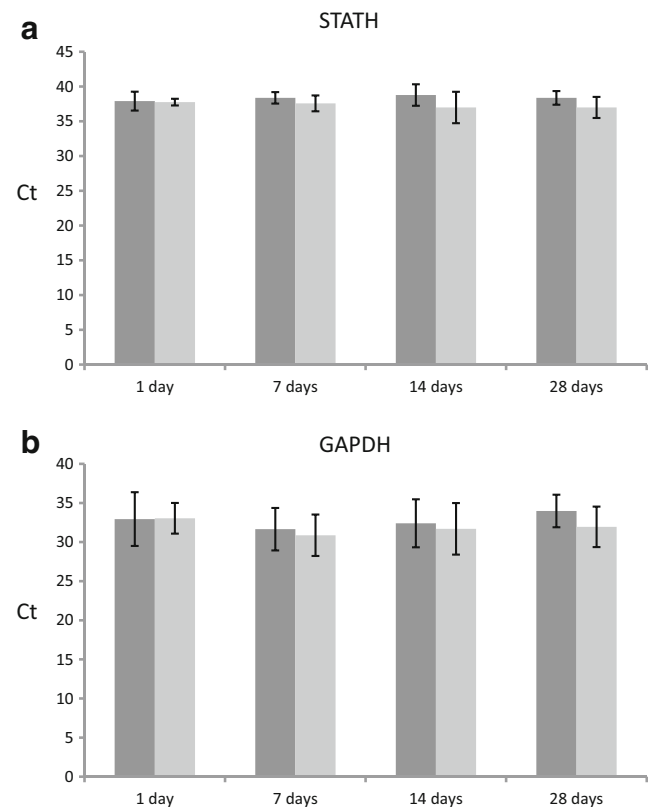


Fig. 1 Mean Ct values of **a** STATH and **b** GAPDH mRNAs in four canine saliva stain samples stored at room temperature for 0, 1, 7, 14, and 28 day(s). The gray bars indicate the Ct values of the four canine saliva stain samples stored at room temperature for 1, 7, 14, and 28 day(s); the light gray bars indicate the Ct values of the corresponding fresh samples. The Ct values of canine saliva stain samples stored at room temperature for 1, 7, 14, and 28 day(s) were not significantly different from those of fresh canine saliva samples (*t* test; $p > 0.05$)

Table 2 The detection of STATH, CA-VI, Canf1, Canf2, and GAPDH mRNAs in various canine and human samples and mock forensic samples

Sample	n	STATH	CA-VI	Canf1	Canf2	GAPDH
Canine saliva	20	19	1	11	4	20
Canine skin surface	20	1	1	1	0	20
Canine urine	20	0	0	0	0	11
Canine blood	10	0	9	0	0	10
Human saliva	20	0	0	0	0	0
Human skin surface	20	0	0	0	0	0
Human blood	20	0	0	0	0	0
Mock forensic sample	5	5	0	1	0	5

development of an mRNA-based method for the identification of canine saliva is important.

We evaluated the utility of STATH, CA-VI, Canf1, and Canf2 mRNAs in the identification of canine saliva in forensic samples. Human STATH mRNA has been applied for the identification of human saliva [13–15] because the STATH gene is expressed in the human salivary gland [21]. However, canine STATH mRNA has not been used for the identification of canine saliva to date. In this study, canine STATH mRNA was detected in canine saliva. Moreover, in canine saliva, STATH mRNA exhibited the highest detection ratio of the mRNAs examined in this study. However, STATH mRNA was also detected in 1 of 20 canine body surface samples. This may be due to the habit of dogs of licking their body to groom or resolve stress, resulting in the presence of STATH mRNA on the body surface. Therefore, this result might not have been a non-specific reaction.

This study also hypothesized that canine saliva could be identified from bite marks on human skin. The detection of canine saliva on human skin may indicate the occurrence of licking or biting. Another possibility is the transfer of saliva from the body surface of the dog to human skin when the dog is touched. However, the transfer of dried saliva from the dog body surface to human skin is presumed to be difficult. Thus, the probability of detecting canine saliva on human skin that has been in contact with a canine body is low. Therefore, the detection of STATH mRNA on human skin suggests that canine saliva adheres to human skin. The findings of this study suggest that STATH mRNA was the most useful of the mRNAs evaluated for the identification of canine saliva. However, according to above description, STATH was not detected in all canine saliva samples. Therefore, the sensitivity of this method should be increased, or other mRNA markers should be investigated in addition to STATH.

CA-VI has been reported to be expressed in the canine salivary gland [18]. However, in this study, because the CA-VI mRNA detection ratio in saliva was low and its expression in canine blood was confirmed, it was found to be unsuitable for the identification of canine saliva. Canf1 mRNA was detected in 11 of 20 canine saliva samples and 1 canine body surface sample. In contrast, Canf2 mRNA was not detected in the other samples though the detection ratio of Canf2 mRNA in canine saliva was low. Because the Canf1 and Canf2 mRNAs exhibited lower sensitivity than STATH mRNA, these mRNAs are not suitable indicators of the presence of canine saliva. GAPDH was detected in all samples in which STATH was detected. Therefore, GAPDH was suggested a suitable marker for canine mRNA extraction.

STATH, CA-VI, Canf1, Canf2, and GAPDH mRNAs did not exhibit cross-reactivity with samples of human origin. Canine mRNA was detected even in the presence of material of human origin in the mock forensic samples. This method may have sufficient sensitivity for real-world investigations

because STATH and GAPDH mRNAs were detected in mock forensic samples; however, their sensitivity was not evaluated in this study.

STATH and GAPDH mRNAs were detected in canine saliva samples stored at room temperature for 28 days, suggesting that these mRNAs were stable. Setzer et al. [22] reported the detection of human STATH mRNA in samples stored dried at room temperature for 180 days. Although mRNA stability was evaluated using only 28-day-old samples, our findings suggest that canine STATH mRNA might be detected in samples of greater age.

In valuable samples such as forensic case work, the RNA/DNA co-extraction method is useful for the identification of body fluid and STR analysis using the same sample [23]. The canine saliva identification method presented in this study can be used in conjunction with the RNA/DNA co-extraction method, suggesting that detection of canine saliva and analysis of canine STRs can be performed using the same sample.

Conclusion

We developed a novel method for the identification of canine saliva based on the detection of canine saliva-specific mRNAs. The results of this study indicate that the detection of STATH mRNA is useful for the identification of canine saliva, and GAPDH is a suitable marker for canine mRNA extraction. This mRNA-based assay was also able to detect canine saliva in mock forensic samples. Moreover, STATH and GAPDH mRNAs were detected in samples stored for 1 month. Therefore, the results suggest that this method is useful for identifying canine saliva during a forensic examination. Combining this method and a bite mark analysis will make dog bite identification more reliable.

Acknowledgments The authors thank Dr. Hiroko Nakazawa (Yamanashi Prefectural Police H.Q.), Dr. Yukari Takeuchi (University of Tokyo), and Ms. Ai Asahi (National Research Institute of Police Science) for collecting the canine samples.

Compliance with ethical standards This study was approved by the Ethics Committee of Juntendo University School of Medicine. Informed consent was obtained from all participants who provided samples.

References

1. Langley RL (2009) Human fatalities resulting from dog attacks in the United States, 1979–2005. *Wilderness Environ Med* 20:19–25
2. Cohrn K, Berman R (2015) The forensic dentist: an overview of expert roles in animal bite-mark investigations. *J Calif Dent Assoc* 43:321–330
3. Eichmann C, Berger B, Reinhold M, Lutz M, Parson W (2004) Canine-specific STR typing of saliva traces on dogs bite wounds. *Int J Legal Med* 118:337–342

4. Wictum E, Kun T, Lindquist C, Malvick J, Vankan D, Sacks B (2013) Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework. *Forensic Sci Int Genet* 7:82–91
5. Kun T, Sacks BN, Ballard RB, Lindquist C, Wictum EJ (2013) Developmental validation of Mini-DogFiler for degraded canine DNA. *Forensic Sci Int Genet* 7:151–158
6. Pomara C, D'Errico S, Jarussi V, Turillazzi E, Fineschi V (2011) Bite mark analysis in a fatal dog pack attack. *Am J Forensic Med Pathol* 32:50–54
7. Santoro V, Smaldone G, Lozito P, Smaldone M, Introna F (2011) A forensic approach to fatal dog attacks. A case study and review of the literature. *Forensic Sci Int* 206:e37–e42
8. Bernitz H, Bernitz Z, Steenkamp G, Blumenthal R, Stols G (2012) The individualisation of a dog bite mark: a case study highlighting the bite mark analysis, with emphasis on differences between dog and human bite marks. *Int J Legal Med* 126:441–446
9. Willott GM (1974) An improved test for the detection of salivary amylase in stains. *J Forensic Sci Soc* 14:341–344
10. Tsutsumi H, Higashide K, Mizuno Y, Tamaki K, Katsumata Y (1991) Identification of saliva stains by determination of the specific activity of amylase. *Forensic Sci Int* 50:37–42
11. Quarino L, Dang Q, Hartmann J, Moynihan N (2005) An elasa method for the identification of salivary amylase. *J Forensic Sci* 50:873–876
12. Mocharla H, Mocharla R, Hodes ME (1990) α -Amylase gene transcription in tissues of normal dog. *Nucleic Acids Res* 18:1031–1036
13. Juusola J, Ballantyne J (2003) Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. *Forensic Sci Int* 135:85–96
14. Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M (2008) Evaluation of mRNA-based approach for identification of saliva and semen. *Legal Med* 11:125–128
15. Haas C, Klessner B, Maake C, Bar W, Kratzer A (2009) mRNA profiling for body fluid identification by reverse transcription endpoint PCR and real-time PCR. *Forensic Sci Int Genet* 3:80–88
16. Kamata Y, Miyanomae A, Nakayama E, Miyanomae T, Tajima T, Hoshi H (2007) Characterization of dog allergens Canf1 and Canf2. *Int Arch Allergy Immunol* 142:291–300
17. Ichihara N, Tsukamoto A, Kasuya T, Shibata S, Nishita T, Murakami M et al (2007) Gene expression of secretory carbonic anhydrase isozymes in striated ducts of canine salivary glands using a laser microdissection system. *Anat Histol Embryol* 36: 357–360
18. Kasuya T, Shibata S, Kaseda M, Ichihara N, Nishita T, Asari M (2007) Immunohistolocalization and gene expression of the secretory carbonic anhydrase isozymes (CA-VI) in canine oral mucosa, salivary glands and esophagus. *Anat Histol Embryol* 36:53–57
19. Murakami M, Kasuya T, Matsuba C, Ichihara N, Nishita T, Fujitani H et al (2003) Nucleotide sequence and expression of cDNA encoding canine carbonic anhydrase VI. *DNA Seq* 14:195–198
20. Kipps AE, Whitehead PH (1975) The significance of amylase in forensic investigations of body fluids. *Forensic Sci* 6:137–144
21. Schlesinger DH, Hay DI (1977) Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva. *J Biol Chem* 252:1689–1695
22. Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. *J Forensic Sci* 53:296–305
23. Haas C, Hanson E, Anjos MJ, Banemann R, Berti A, Borges E et al (2013) RNA/DNA co-analysis from human saliva and semen stains—results of a third collaborative EDNAP exercise. *Forensic Sci Int Genet* 7:230–239