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Prevalence and persistence of male DNA identified in mixed saliva samples after intense kissing

Natália Kamodyová [a,*], Jaroslava Durdiaková [a,b], Peter Celec [a,c,d], Tatiana Sedláčková [a], Gabriela Repiská [a], Barbara Sviežená [e], Gabriel Minárik [a,c,f]

[a] Institute of Molecular Biomedicine, Comenius University, Sasinkova 4, 81108 Bratislava, Slovakia
[b] Institute of Physiology, Comenius University, Sasinkova 2, 81108 Bratislava, Slovakia
[c] Department of Molecular Biology, Comenius University, Mlynská dolina, 84215 Bratislava, Slovakia
[d] Institute of Pathological Physiology, Comenius University, Sasinkova 4, 81108 Bratislava, Slovakia
[e] Institute of Forensic Science, Department of Criminalistic Biology and Genetic Analysis, Sklabinská 1, 81272 Bratislava, Slovakia
[f] GENETON Ltd, Cabanova 14, 84102 Bratislava, Slovakia

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ABSTRACT

Identification of foreign biological material by genetic profiling is widely used in forensic DNA testing in different cases of sexual violence, sexual abuse or sexual harassment. In all these kinds of sexual assaults, the perpetrator could constrain the victim to kissing. The value of the victim's saliva taken after such an assault has not been investigated in the past with currently widely used molecular methods of extremely high sensitivity (e.g. qPCR) and specificity (e.g. multiplex Y-STR PCR).

In our study, 12 voluntary pairs were tested at various intervals after intense kissing and saliva samples were taken from the women to assess the presence of male DNA. Sensitivity-focused assays based on the SRY (single-copy gene) and DYS (multi-copy gene) sequence motifs confirmed the presence of male DNA in female saliva after 10 and even 60 min after kissing, respectively. For specificity, standard multiplex Y-STR PCR profiling was performed and male DNA was found in female saliva samples, as the entire Y-STR profile, even after 30 min in one sample.

Our study confirms that foreign DNA tends to persist for a restricted period of time in the victim's mouth, can be isolated from saliva after prompt collection and can be used as a valuable source of evidence.

1. Introduction

In forensic genetic profiling, it is necessary to isolate and analyse DNA from various biological materials and to compare the DNA profiles from hundreds of samples in order to identify the crucial one [1,2]. The main objectives of this investigation are body fluids [3]. Saliva and saliva-stained material have proven to be good and valuable sources of DNA for genotyping in certain forensic settings [4]. Physical contact by means of a sexual act or kissing results in the transfer of DNA from one individual to another [5], which could be used for personal identification of concerned persons, providing valuable evidence and intelligence information to police investigations [6].

Correct DNA quantification is an essential aspect of obtaining reliable results in subsequent genotyping analysis. Various commercial kits enable forensic laboratories to simultaneously obtain a quantitative and qualitative assessment of total human DNA in a single, highly sensitive real-time PCR reaction [7]. For the detection and quantification of male chromosomal DNA in forensic samples, Y-chromosome sequence-based assays are widely used (e.g. the SRY-based Quantifiler® Y human male DNA quantification kit, Plexor® HY System for simultaneous detection of total human and male DNA, and DYS14-based home-made assays for the detection of foetal gender from the plasma of pregnant women). For individual identification, multiplex STR profiling is a well-established, convenient and low-cost approach for unambiguous characterisation of different loci in the human genome and provides a reference standard to detect and resolve the probative profile in DNA-based forensic testing [8]. For specific analyses in cases of sexual assaults, where mixed male:female samples are analysed, Y-chromosome STR profiling enables the unambiguous identification of male DNA. Y-STR testing is used preferentially as an additional test or when quantity of male DNA is insufficient for

* Corresponding author at: Natália Kamodyová, Institute of Molecular Biomedicine, Comenius University, Sasinkova 4, 81108 Bratislava, Slovakia.
Tel.: +421 2 59357371; fax: +421 2 59357631.
E-mail address: n.kamodyova@gmail.com (N. Kamodyová).

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autosomal STR analysis [9]. In this field, the most commonly used commercially available kits, such as Y-STR multiplex systems from Promega (PowerPlex® Y) and Applied Biosystems (YFiler®), are well-accepted in forensic laboratories.

In cases where samples are collected from a living victim as evidence material (questioned sample), the crucial factor that can influence the success of analysis is the time between the criminal act and sample collection. Spermatozoa, which can be used for DNA isolation and individual identification, have been detected in swabs taken from the oral cavity up to 8 h after a sexual assault [10]. Banaschak et al. [5] reported that male DNA transferred through intense kissing persists in female saliva for a very short time (max. 60 s). However, the methods used in that study were based on home-made STR profiling assays with three autosomal and one Y-chromosomal marker and with the sequencing of mitochondrial DNA. The current methods used for forensic analysis focused on male:female mixed samples surpass previously used assays in sensitivity as well as specificity.

The aim of our study was to analyse mixed saliva samples after intense kissing to prove the possibility of detection and the time of persistence of male DNA in female saliva samples employing currently used molecular methods. This may be interesting for forensic experts and criminalists, as it brings a new information about the possibility of using saliva evidence in such cases and helps to estimate the crucial time period for the detection of transferred DNA remains in a mixed saliva sample after contact between the victim and the perpetrator.

2. Materials and methods

Volunteers involved in our study were informed in detail about the concept of the study and signed informed consent for participation. A total of twelve heterosexual pairs participated in the experiments. Each pair was instructed to kiss intensively for at least 2 min to stimulate maximum mouth-to-mouth carry-over of saliva. Females were asked to collect 2 mL of whole saliva by spitting. Saliva samples were collected before kissing, as soon as possible after kissing (max. 1 min) and at 5, 10, 30 and 60 min intervals during which they abstained from eating and drinking. Saliva samples were centrifuged for 10 min at 5000 × *g* and the pellet was used for DNA isolation. The isolation procedure was based on the silica membrane QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) and the original manufacturer's protocol for DNA purification from blood/body fluids was used. Total DNA, extracted from women saliva collected before and after kissing and from male reference saliva, was quantified using a Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). Male samples for DNA identity testing using Y-STR system AmpFLSTR® Yfiler® were collected using cotton swabs. DNA from cotton swabs was extracted using a Chelex®100 (Biorad, Hercules, USA) based method [11].

The Y-chromosome-specific qPCR assays based on single-copy SRY and multiple-copy DYS-14 sequences were used for the detection and quantification of male DNA in female saliva. Real-time PCR was performed on a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) using 1 μL of template DNA in a 15 μL reaction volume containing final concentrations of 1x QuantiFast Probe Master Mix (Qiagen, Hilden, Germany), 0.72 μmol/L of each primer and 0.42 μmol/L of the fluorescent probe. The following PCR programme was used: initial denaturation step at 95 °C for 3 min, followed by 50 cycles each consisting of denaturation at 95 °C for 3 s and combined annealing/extension at 60 °C for 30 s. The primer and probe sequences were as follows:

SRY-F (5′-GCCATTTTTCGGCTTCAGTA-3′).
SRY-R (5′-CGGAGAAGCTCTTCCTCCCT-3′).
SRY-PROBE (5′-FAM-TCCCAGAATGCGAAACTCAGA-TAMRA-3′) [12].
DYS14-F (5′-GGGCCAATGTTGTATCCTTCTC-3′).
DYS14-R (5′-GCCCATCGGTCACTTACACTTC-3′).
DYS14-PROBE (5′-FAM-TCTAGTGGAGAGGTGCTC-TAMRA-3′) [13].

Amplicon sizes were 238 and 84 bp for SRY and DYS, respectively. The data from SRY qPCR were converted to genome equivalents (GEs) by the use of a standard factor of 6.6 pg [13]. Because of copy variation of DYS region in human genome qPCR data for this assay were not converted to genome equivalents.

For confirmation of the origin of Y-chromosomal sequences in female saliva, the Y-STR system (AmpFLSTR® Yfiler® PCR Amplification Kit, Applied Biosystems, Foster City, USA) was used according to the manufacturer's instructions. Capillary electrophoresis and genotyping was performed on an ABI 3100-Avant Genetic Analyzer using GeneMapper v3.5 software (Applied Biosystems, Foster City, USA). The minimum peak height for allele calling was set to "Automatic".

3. Results

The concentration of DNA extracted from saliva samples using the silica membrane-based kit had a median of 22.23 ng/μL (mean 42.2 ng/μL, range 3.34–233.16 ng/μL). The yield of DNA extracted from saliva samples collected at various time points after kissing did not differ significantly.

The SRY real-time PCR system allowed the detection of male DNA in female saliva samples a maximum of 10 min after kissing. In samples taken 1 min after kissing, 223.8 ± 369.7 GEs of male DNA was found in 11 out of 12 female saliva samples. After 5 and 10 min, 123.1 ± 159.1 GEs and 38.7 ± 109.5 GEs of male DNA was present in 4 out of 12 female saliva samples. Genome equivalents for all samples calculated according to the SRY assay are summarised in Table 1. The DYS assay allowed for the detection of male DNA in the saliva of women collected even 60 min after kissing. DYS qPCR detected male DNA in all female saliva samples taken 1 min after kissing. Male DNA was still found in 11 out of 12 female samples after 5 and 10 min, in 10 out of 12 samples after 30 min and in 8 out of 12 samples after 60 min (data are summarised in Table 2). In the SRY assay, the mean C_t values for male DNA amplification from female saliva samples taken 1, 5 and 10 min after kissing were 31.9, 33.3 and 34.8, respectively. The mean C_t values for the DYS assay after male DNA amplification from female saliva samples taken 1, 5, 10, 30 and 60 min after kissing were 25.1, 28.9, 29.4, 31.9 and 33.0, respectively (Fig. 1). The SRY and DYS assays diverged, on average, by 5.5 C_t.

Table 1
Genome equivalents of male DNA present in female saliva samples after kissing estimated according to the SRY assay.

Sample	Saliva samples – minutes after kissing				
	1	5	10	30	60
A	78.7	–	–	–	–
B	116.6	14.3	4.0	–	–
C	1128.9	126.7	4.2	–	–
D	47.6	–	–	–	–
E	122.4	–	–	–	–
F	3699.0	346.9	330.6	–	–
G	36.3	–	–	–	–
H	645.6	4.6	9.6	–	–
I	–	–	–	–	–
J	23.7	–	–	–	–
K	10.5	–	–	–	–
L	28.0	–	–	–	–
Mean ± SD	223.8 ± 369.7	123.1 ± 159.1	38.7 ± 109.5	–	–

Table 2
C_t values for the DYS assay obtained after male DNA amplification from female saliva samples after kissing.

Sample	Saliva samples – minutes after kissing				
	1	5	10	30	60
A	25.8	30.1	31.3	31.9	–
B	24.4	26.8	28.4	30.3	33.9
C	24.1	27.2	28.5	32.4	34.3
D	25.7	28.8	28.0	30.6	33.5
E	23.2	28.2	27.8	28.9	30.2
F	19.5	24.9	23.6	31.0	31.8
G	24.8	29.8	30.2	32.8	34.0
H	23.7	27.9	30.5	34.2	32.2
I	27.8	33.4	33.3	–	–
J	25.9	30.4	31.3	34.2	–
K	28.3	–	–	–	–
L	27.4	31.1	31.0	32.4	34.2
Mean ± SD	25.1 ± 2.4	28.9 ± 2.3	29.4 ± 2.6	31.9 ± 1.7	33.0 ± 1.5

The multiplex STR system Yfiler® was used to compare male DNA from cotton swabs with DNA profiles from female saliva after kissing (Fig. 2). Profiles from the Y-STR system confirmed the presence of specific male DNA in saliva samples from women and allowed the detection of residual male DNA. Full Yfiler® profiles were acquired in four samples after 10 min and in one sample 30 min after kissing. The correctly identified male allele counts in the entire tested group of samples decreased with time from 203, 196, 153, 127, 66 and 20 in the time interval of 1, 5, 10, 30 and 60 min after kissing, respectively. The complete results of the identified allele counts in all samples are summarised in Table 3.

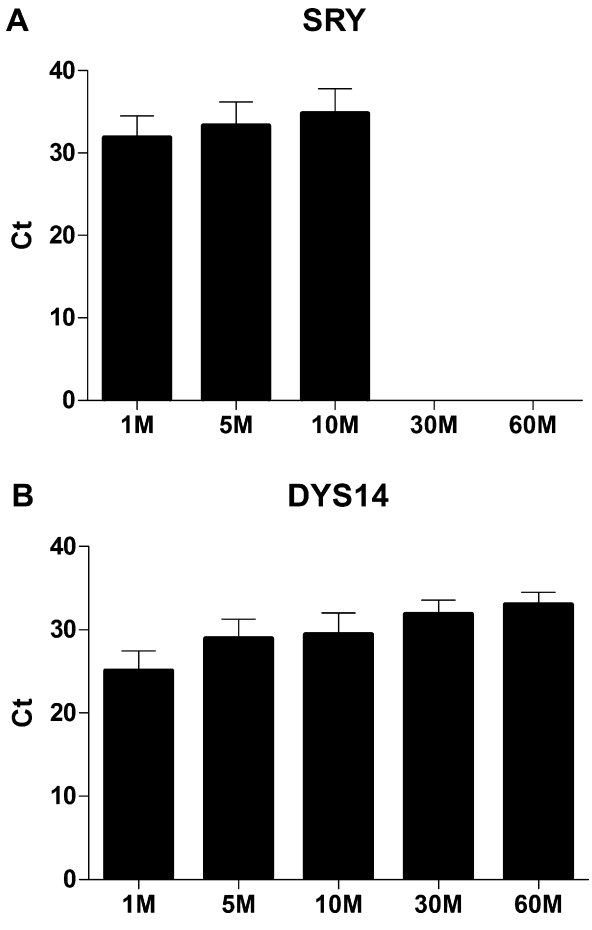

Fig. 1. Mean C_t values of male DNA obtained from female saliva collected at different intervals after kissing. Two different protocols were used: (A) SRY single-copy sequence allowed the detection of male DNA after 1, 5 and 10 min after kissing and (B) amplifying multi-copy sequence DYS14 enabled the detection of male DNA after 1, 5, 10 and 30 min and even after 60 min.

4. Discussion

Biological evidence is one of the most common types of evidence found at a crime scene. Primary sources of DNA samples, specifically, blood, semen and saliva are generally either involved in the act of committing the crime, or lost in an involuntary and possibly unavoidable manner. All of the following sources of forensic evidence can provide useable DNA samples for testing. Fingernail scrapings and clippings are routinely examined for the presence of foreign DNA, especially in cases of struggles, homicides and sexual assaults. Previous research has confirmed that the prevalence of foreign DNA beneath fingernails in the general population is low and tends not to persist for an extensive period of time (only 7% of samples gave a signal with >5 STR markers) [14]. This low prevalence and short persistence enables the use of material taken from beneath of the nails for specific forensic analysis were the victim actively defended during the crime act, because there is only a limited chance of finding false genotypes. Moreover, the short persistence was detected only in samples taken from living people and not in cases when DNA was isolated from homicide victims. In this kind of sample, the suspect's DNA was detected in more than 25% of tested samples and 50% of these samples were high-level mixed samples, so the full profile of the suspect was clearly recognised [15]. Similar to foreign DNA identified beneath the nails, kissing provides a direct source of foreign DNA contamination if saliva samples are used for forensic analysis after constrained kissing [5]. From a different point of view, the introduction of male DNA into female saliva can be an indicator of voluntary sexual contact, usually represented by kissing, in specific cases. In Banaschak's paper, with a similar study scheme to ours, it was found that contamination of saliva samples by intense kissing is possible but restricted to a very short time period immediately following kissing (1 min) [5]. However, this study was performed almost 15 years ago and currently used methods have much higher sensitivity and specificity. Therefore, we decided to mimic the study design and to test currently used methods for mixed DNA detection, quantification and specification. In our study, we tried to test for the presence and persistence of male DNA in female saliva using a two-step approach: in the first step, only the presence of male DNA was detected and quantified by qPCR, and in the second step, the authenticity of the male DNA signal was confirmed with the Yfiler® multiplex STR genotyping system, which is widely used in forensic laboratories. Our results contrast those previously published by Banaschak et al. [5], as we were able to detect male Y-chromosomal sequences persisting in the women's mouths even 60 min after kissing. For male DNA detection, Y-chromosome based assays are routinely used and assays based on the SRY-genomic region represent the gold standard for such testing. Therefore, in the first step of the analysis, we used an SRY-based assay for male DNA detection, as this sequence is present on the Y-chromosome in one copy. But, as reported previously by Zimmermann et al. [13], much higher sensitivity can be obtained by amplifying multiple-copied sequences. The detection of male DNA by the DYS14 assay had a 10-fold lower detection limit (0.4 genome equivalents) than did measurements of SRY [13]. Therefore, the DYS14 protocol, with higher sensitivity, was also used to detect male-specific DNA in our study. The DYS14 assay demonstrated the persistence of male DNA in female saliva samples even 60 min after kissing. On the other hand, the interindividual variability in copy number of DYS14 genomic sequences complicated the estimation of genome equivalents of male DNA which were present in the tested samples. For this reason, we used the SRY assay for the estimation of genome equivalents. The genome equivalent counts found in saliva samples

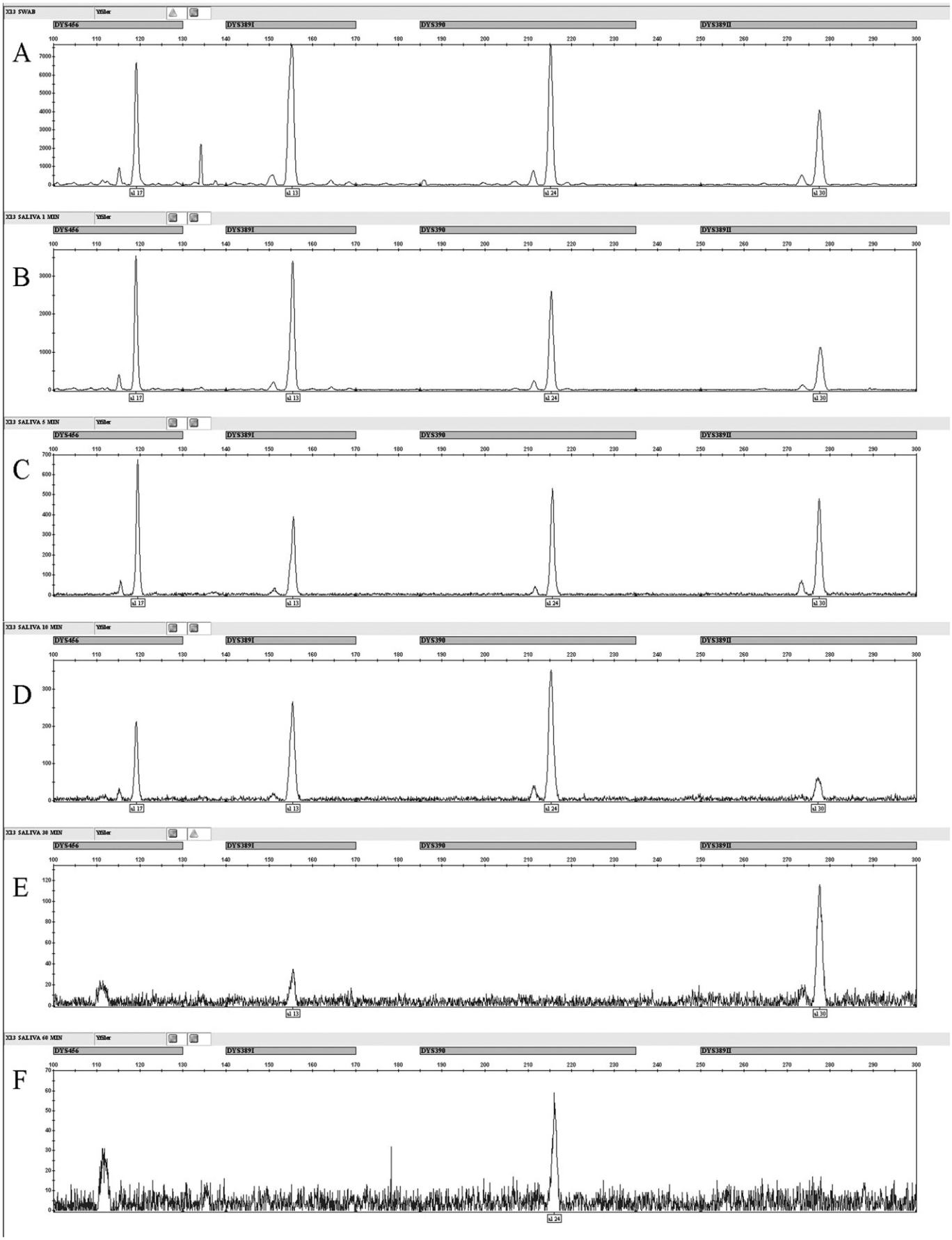

Fig. 2. Electropherograms of Yfiler® amplification of residual male DNA from female saliva samples taken at different intervals after kissing. (A) Positive control swab from the man and (B–F) signals from amplified DNA isolated from female saliva taken 1, 5, 10, 30 and 60 min after kissing, respectively.

Table 3
Summary of identified Y-STR allele counts genotyped correctly in all tested samples at different time points after kissing.

Sample	Swab	Saliva samples – minutes after kissing				
		1	5	10	30	60
A	17	17	11	8	0	0
B	17	17	17	15	12	1
C	17	17	17	17	6	3
D	17	17	17	17	4	0
E	16	16	16	14	14	4
F	17	17	17	17	17	6
G	17	15	12	8	2	1
H	17	17	17	17	5	3
I	17	14	3	1	0	0
J	17	17	11	5	2	2
K	17	15	1	0	0	0
L	17	17	14	8	4	0
Sum	203	196	153	127	66	20

correlated well when the results from the SRY-based assay were compared with Y-STR profiling. So, SRY-based or possibly also DYS-based male DNA absolute or relative quantification can be used before Y-STR typing to test the applicability of the use of saliva for forensic testing.

To confirm the specificity in our tests, we analysed our samples with a multiplex Y-STR system, AmpFLSTR® Yfiler® PCR Amplification Kit (Applied Biosystems, Foster City, USA). We were able to determine the full Yfiler® profile in four samples (33.33% of tested samples) 10 min after kissing. In one sample, it was possible to get the full profile even after 30 min. This shows that saliva samples, or mouth washes in specific cases, could be of great value in forensic analysis. In our study, relatively high variability was detected in genome equivalent counts. This can be explained by various intensities of kissing and subsequently different mouth-to-mouth carry-over of saliva among pairs, which is not possible to control exactly. The time of persistence also depends on the salivation of the women, which could be highly individually variable. However, these limits could have less influence in cases where the victim dies during or shortly after the assault. According to the previously published results of a retrospective study [15] performed on material from beneath the nails and from nails directly taken from the homicide victims mentioned above, we could predict the possibility of the detection DNA remains from the perpetrator in the mouth of the victim in cases of murder after sexual assault at intervals much longer than the 30–60 min presented in this study. In such cases, the perpetrator's DNA can be detected in a simple mouth wash taken from the victim, as the victim's salivation has stopped. In our study cellular fraction from collected salivary samples was used for DNA isolation. Therefore, if mouthwash should be starting material for forensic sample processing, concentration of material used for DNA isolation with simple centrifugation step is possible. So the volume of solution used for mouthwash could be relatively large which could higher the probability of successful analysis and lower the probability of false negative results in such cases. On the other hand if the continuing salivation of the living victim should dilute out the perpetrator's DNA, the victim could spit the saliva in some container as soon as possible, and this could be used as further evidence of a criminal act. Moreover if the spit will be performed on some absorbent and this will be dried out the evidence should remain usable for forensic testing much longer time.

One of the drawbacks of our study is the sampling time limited to 60 min after kissing. As mentioned in the introduction one of the crucial factors influencing the success of forensic analysis is the time between the criminal act and the sample collection. Since there are not many crime scene samples which are taken within 1 h after criminal act and since we were able to detect male DNA in most of the female saliva even 60 min after kissing it would be beneficial to extend the sampling time in further studies. Moreover, it could be of high importance to test if the male DNA persist in mouth of victim longer in cases when the victim has not survive the attack as was mentioned above. Another drawback is related to the Y-STR evidence. It has to be noted that this evidence is not a unique identifier. It is possible for an unrelated person to have the same Y-STR profile, purely as a product of chance. Therefore study focused on the autosomal STR profiling with much higher discrimination power should be the next step in our research. Such additional studies could bring new and valuable information with relevant information and immediate impact in the field of forensic sciences.

Taken together, we have confirmed that kissing results in mouth-to-mouth DNA carryover, which can be valuable for forensic testing.

5. Conclusion

In our experimental setup, we were able to determine the presence of male DNA in female saliva even after 60 min with a simple qPCR-based test. Moreover, in more than one third of samples, it was possible to get the full Y-STR genotype of the relevant male 10 min after kissing. The study was conducted on volunteers but the results are applicable in different cases of sexual assaults in which perpetrator forces the victim to kissing. Our data clearly indicate the possibility of using saliva as a source of evidence of sexual harassment, infidelity, rape or other kinds of sexual assaults in cases when a biological sample can be collected within a short time after the act.

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